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PURIFICATION AND SOME PROPERTIES OF HUMAN TISSUE ALKALINE PHOSPHATASES

by

© JOAN MARGUERITE TREPANIER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled PURIFICATION AND SOME PROPERTIES OF HUMAN TISSUE ALKALINE PHOSPHATASES

submitted by JOAN MARGUERITE TREPANIER

in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

in MEDICAL LABORATORY SCIENCE

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(Supervisor)

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Date..April..28,..1975

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ABSTRACT

The roles proposed for alkaline phosphatase in phosphate transport across membranes, ossification of bone tissue and removal of pyrophosphate from nucleation sites in the matrix of the bone, as well as increased serum enzyme activity in various disease processes, justify research activities that have been directed towards its purification and characterization. The present study was undertaken to purify human alkaline phosphatases and to study certain properties of the enzyme.

Alkaline phosphatase was extensively purified from human liver tissue and partially purified from human bone, intestine and placenta by butanol extraction, ion exchange chromatography, gel permeation chromatography, affinity chromatography and ammonium sulfate fractionation. The use of affinity chromatography with concanavalin A-Sepharose was developed to purify alkaline phosphatase. The enzyme from the liver was purified to a specific activity of 480 U/mg protein and was estimated to be 75% alkaline phosphatase protein. The extensively purified liver alkaline phosphatase was electrophoresed on polyacrylamide disc gels and found to be essentially pure with the single band of enzyme activity coinciding with the major band of protein revealed by Coomassie Blue staining. Stability studies were carried out to determine the most favorable

storage conditions for the purified alkaline phosphatase.

The purified or partially purified isoenzymes from liver intestine, bone, and placenta were subjected to isoelectric focusing on polyacrylamide disc gels in order to determine their isoelectric points in relation to the isoelectric points of the components found in normal and pathological sera. The focusing technique was found to be superior to electrophoresis for the separation of the four isoenzymes of alkaline phosphatase. Polyacrylamide disc gel isoelectric focusing was used to determine the following isoelectric points: liver tissue - 4.0; liver serum - 4.2; bone - 4.3, 4.4, 4.5; placenta - 4.3, 4.6; intestine - 4.7, 4.8.

The purified liver enzyme and appropriate standards were subjected to gel permeation chromatography and sodium dodecyl sulfate disc gel electrophoresis and the respective molecular weights were determined. The apparent molecular weight was found to be 220,000 (range: 199,000 - 226,000) daltons and the subunit molecular weight to be 76,700 daltons.

The relative specificities of the isoenzymes for four substrates with different chemical composition were investigated. The isoenzymes were most active towards p-nitrophenyl phosphate, followed by β-naphthol phosphate, β-glycerophosphate and phenolphthalein phosphate.

Experiments on heat stability of the isoenzymes were carried out. Bone was least stable to heat, placenta and intestine the most stable and liver was moderately heat stable.

The liver and bone isoenzymes were treated with the enzyme neuraminidase to cleave the terminal sialic acid residues. The desialated isoenzymes were shown to maintain full enzymatic activity. Desialation resulted in changes in the isoelectric points to 6.6 and 6.8. The desialated liver enzyme had an apparent molecular weight of 188,000 (range: 175,000 - 193,000) as determined by gel permeation chromatography on Sephadex G-200.

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LIST OF ABBREVIATIONS

AMP	2-amino-2-methyl-1-propanol
CNBr	cyanogen bromide
con A	concanavalin A
DEAE-cellulose	diethylaminoethyl cellulose
EDTA	ethylenediaminetetraacetate
I.D.	internal diameter
KCN	potassium cyanide
MES	2.(morpholino) ethanesulfonic acid
nm	nanometer
pH_I	isoelectric point
SDS	sodium dodecyl sulfate
TEA	triethanolamine
TES	N-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid
Tris	tris(hydroxymethyl)aminoethane
U	international unit
°	degree, expressed in Centigrade units

CHAPTER I

REVIEW OF THE LITERATURE

Alkaline phosphatase has been the subject of extensive research in recent years and several reviews have been presented on the achievements in the field (Fernley, 1971; Kaplan, 1972).

A. Type of Activity and Reaction Catalyzed by Alkaline Phosphatase

1. Monoesterase Activity

Alkaline phosphatase (orthophosphoric monoesterase phosphohydrolase, EC 3.1.3.1) catalyzes the reaction involving the hydrolysis of phosphomonoesters, having a pH dependence for their activity on the alkaline side of neutrality. The enzyme has a broad range of substrate specificity. It will hydrolyze hexosephosphates, nucleotides, glycerophosphates, ethyl phosphate and phenyl phosphate (Kay, 1932; Harkness, 1968). It had been reported that purified extracts of mammalian tissues have appreciable pyrophosphatase activity (Moss et al, 1967) as well as adenosine triphosphatase and fluorophosphatase activities (Fernley and Walker, 1967). Proof of related monoesterase and pyrophosphatase activities is based on heat inactivation studies and the inhibition of pyrophosphatase activity by L-phenylalanine, a specific inhibitor of intestinal alkaline phosphatase (Moss et al, 1969).

The reaction catalyzed by alkaline phosphatase

is as follows:

where RO- represents the backbone of an alcohol compound.

2. Transferase Activity

There is also evidence that alkaline phosphatase may function as a transferase if there is a phosphate acceptor present. This usually results in increased enzyme activity which is attributed to transphosphorylation in which the net transfer of phosphate from substrate to the hydroxyl group of the buffer is more rapid than transfer of the phosphate group to water (hydrolysis). For significant transphosphorylation to occur (Wilson, Dayan and Cyr, 1964) the phosphate acceptor must contain a hydroxyl group and either a second hydroxyl group or an amino group adjacent to the accepting hydroxyl group. Studies of the transferase action of milk and intestinal alkaline phosphatases have shown that compounds such as glucose, glycerol and propandiol can accept a phosphoryl residue from a wide variety of donors (Morton, 1958). The general equation of transferase activity would be:

where R is the donor and R' the acceptor of phosphate.

B. Distribution of Alkaline Phosphatase

Alkaline phosphatase is found in many mammalian species and is abundant in those tissues concerned with the transport of nutrients and in developing tissues. Alkaline phosphatase activity has been found in the following tissues: intestinal mucosa, placenta, kidney, bone, liver, lung, spleen as well as white blood cells. It is also found in bile but here it has been suggested to be derived from the liver which clears alkaline phosphatase to the bile (Bode, Zelder and Neuberger, 1973).

1. Within the Tissues

Alkaline phosphatase is not homogeneously distributed within the tissues. Intestinal alkaline phosphatase is located predominantly at the surface membrane of the epithelial cell microvilli (Clark, 1961; Ito, 1969). The placental enzyme is located at the surface of the trophoblastic syncytium (Wachstein, Meagher and Ortiz, 1963). The brush border of the proximal tubule epithelial cells is the site of alkaline phosphatase in the kidney. In bone, alkaline phosphatase is found in the hypertrophic cartilage cells, osteoblasts and osteocytes (Ali, Sajdera and Anderson, 1970) while alkaline phosphatase in liver tissues is found in the membranes adjacent to the bile cannaliculi (Wachstein and Meisel, 1957) and the sinusoidal surfaces of the liver (Picardi,

Gardiol and Gautier, 1967). It is almost absent from muscle, mature connective tissue, nonossifying cartilage and red blood cells.

2. Cellular Localization

Both light and electron microscopic studies have shown that mammalian alkaline phosphatases are primarily found at the absorptive or secretory surfaces of cells (Reale, 1962; Goldfischer, Ersner and Novikoff, 1964; Mizutani and Barrnett, 1965). They are bound to membrane fractions as shown when cell homogenates are subjected to high speed centrifugation (Morton, 1954; Emmelot et al, 1964). Recent studies on bone alkaline phosphatase show it to be located on the outer membrane of bone cells (Gothlin and Ericsson, 1971).

C. Methods for the Purification of Alkaline Phosphatase

Alkaline phosphatase is a membrane bound enzyme requiring extensive extraction techniques.

1. Butanol Extraction Procedure

The classic procedure for extracting alkaline phosphatase activity from animal tissue with butanol was introduced by Morton (1950). Modification of this procedure by Moss et al (1967) involved extracting the enzyme with butanol, fractionation with acetone and ammonium sulfate, followed by Sephadex gel permeation and anion exchange chromatography. By this technique workers were

able to obtain a low yield of a partially purified preparation. Smith et al (1968) further modified this technique to include large scale recycling gel filtration. By this method they obtained a 7.4% yield of human liver alkaline phosphatase with a specific activity of 165 units per mg protein and a 10.5% yield of human intestinal alkaline phosphatase with a specific activity of 136 units per mg protein.

2. Trichlorotrifluorethane Extraction Procedure

Narayanan and Appleton (1972a) introduced the use of trichlorotrifluorethane (Genetron-113) for extraction of alkaline phosphatase followed by the use of Sephadex gel permeation and anion exchange chromatography. They obtained a preparation of human intestinal alkaline phosphatase of specific activity 54 units per mg protein. However, 80% of the initial activity of the homogenate in the Genetron-113 step is lost compared to a control preparation they did of the same proportions by butanol extraction. The fewer steps involved in the Genetron-113 extraction and purification procedure do not appear to justify this kind of a loss in enzyme activity. Genetron-113 extraction would appear to be too harsh a method for membrane bound proteins especially alkaline phosphatase.

3. Isoelectric Focusing

A method has been described (Usategui-Gomez,

Yeager and Tarbutton, 1974) for the purification of alkaline phosphatase from human placenta using preparative scale isoelectric focusing. The tissue was first homogenized in buffer, fractionated with ammonium sulfate, extracted with butanol and chromatographed on anion exchange column. The preparation was then subjected to isoelectric focusing in a sucrose gradient. See I.D.2. (b) below for further discussion of isoelectric focusing. A 11% yield of alkaline phosphatase was obtained that had a specific activity of 330 units per mg protein.

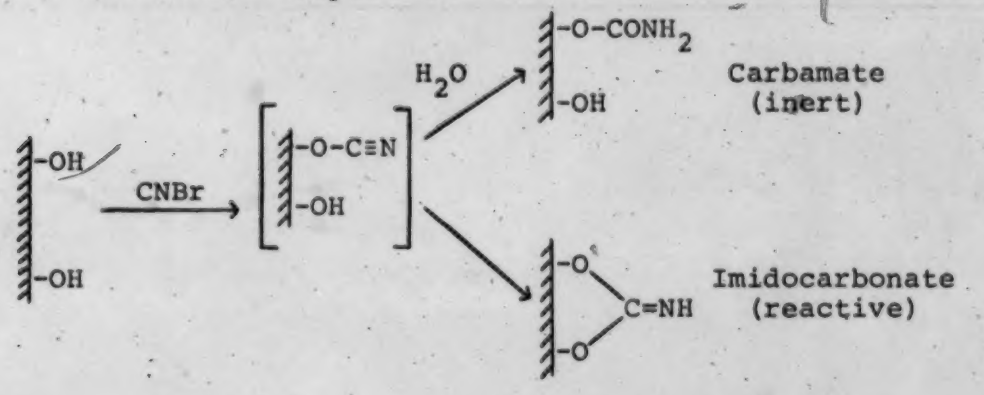
4. Affinity Chromatography Procedure

Affinity chromatography which is a powerful method for purifying macromolecules, exploits the natural specificity of biological interactions. Guilford (1973) gives a review of the chemical aspects of affinity chromatography and the more biological aspects are reviewed by Cuatrecasas and Anfinsen (1971). Affinity chromatography is a type of adsorption chromatography in which the bed material has a specific affinity for the substance to be isolated. The substance to be isolated is absorbed from the solution by the binding component and can be desorbed by changing the experimental conditions after any unbound material is washed from the cclumn. Thus, affinity chromatography isolates substances on the basis of their biological interactions. The support matrix must be physically

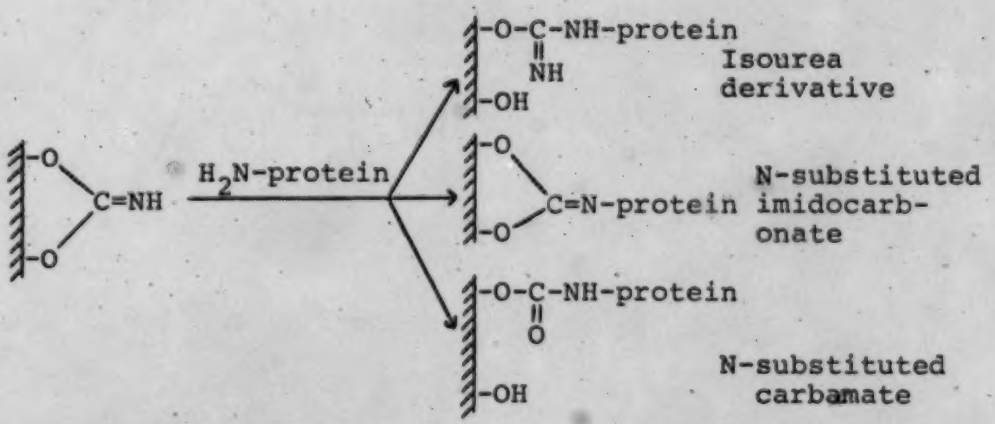
and chemically stable under experimental conditions, it must allow satisfactory column flow rate, and must be capable of slight chemical modification without undergoing structural changes. Sepharose, a beaded agarose polymer, offers such a matrix. The ligand, a biologically suitable binding protein or other material for the isolation of the desired substance, is covalently bound to the sepharose matrix. The most common procedure involves cyanogen bromide activation of the carrier and subsequent coupling of a primary aliphatic or aromatic amine of the ligand to the activated matrix (Axen, Porath and Ernback, 1967).

Concanavalin A (Con A) is a haemagglutinating protein isolated from jack bean. It forms insoluble complexes with polysaccharides and glycoproteins. The binding site of Con A is specific for α-D-mannosyl, α-D-glucosyl and sterically similar residues. Con A can be specifically bound to Sepharose by the cyanogen bromide (CNBr) method. CNBr reacts with hydroxyl groups of Sepharose to form imidocarbonate and carbamate groups. Con A is subsequently coupled to the active product by the imidocarbonate groups reacting with the amino groups on the Con A molecule thus forming Con A-Sepharose.

Activation Step:



Coupling Step:



Con A-Sepharose has been used to purify several glycoproteins: human α-fetoprotein (Caron et al, 1973), very low density lipoprotein (Shore and Shore, 1973) and glycoprotein surface receptors for Con A (Allan, Auger and Crumpton, 1972).

D. Properties of Alkaline Phosphatase

1. Physical Properties

(a) Measurement of Alkaline Phosphatase Activity

(i) Assay Techniques

Typical substrates of alkaline phosphatase include phosphate esters of primary and secondary alcohols, sugar alcohols, cyclic alcohols, phenols, naphthols and nucleoside monophosphates. The analytical methods for measuring alkaline phosphatase activity are of two types: The first measures the amount of phosphate liberated and the second measures the amount of alcohol liberated. The first substrate used to assay alkaline phosphatase was β-glycerophosphate (Bodansky, 1933). The phosphate was measured by the classical Fiske-Subbarow procedure (1925) in which inorganic phosphate is allowed to react with ammonium molybdate at acid pH. The phosphate is measured before and after the serum is incubated with the substrate. The newly formed phosphomolybdate is then reduced by 1,2,4-amino-naphtholsulfonic acid to yield a blue colored product. The absorbance of this complex can be measured at 600 nm.

King and Armstrong (1934) developed the first clinical method based on the amount of liberated alcohol in which phenol split from phenyl phosphate was measured. Later methods developed in which the free alcohol was itself chromogenic, requiring no reagents

for color development and making it possible to continuously record the enzymatic activity on a recording spectrophotometer (Bowers and McComb, 1966). Such chromogenic substrates include p-nitrophenyl phosphate (Bessey, Lowry and Brock, 1946), phenolphthalein diphosphate (Klein, Read and Babson, 1960). Newer substrates β-naphthyl phosphate (Greenberg, 1962) and 4-methylumbelliferyl phosphate (Fernley and Walker, 1965) have now been synthesized whose hydrolysis products fluoresce and thus are detectable in minute quantities. These methods are of no clinical advantage however since spectrophotometric methods can adequately detect normal serum levels of alkaline phosphatase.

(ii) Substrate Specificity and the Effect of the Composition of the Buffer on the Assay

When different methods are used by various workers for measuring the activity of alkaline phosphatase, it is difficult to obtain a comparison of the total serum levels since the isoenzymes of alkaline phosphatase have different substrate specificities (Landau and Schlamowitz, 1961; Wolf, Dinwoodie and Morgan, 1969). However, a common choice of substrates does not necessarily lead to uniformity. The use of different buffers must also be taken into account when comparing activities of the isoenzymes (Haije, 1973). The original buffer used in the p-nitrophenyl phosphate assay of alkaline phosphatase was

glycine (Bessey, Lowry and Brock, 1946). The glycine buffer was later found to be inhibitory (Ahmed and King, 1960). Bowers and McComb (1966) modified this method using 2-amino-2-methyl-1-propanol (AMP) as the buffer. The use of such an amino alcohol buffer results in marked accentuation of enzyme activity because the alcohol groups on the buffer molecules act as acceptors of the liberated phosphate resulting in the measurement of the transferase activity of alkaline phosphatase.

(b) Structure and Molecular Weight of Alkaline Phosphatase.

Most of the work on the structure of human alkaline phosphatase has been carried out on the placental isoenzyme. Ghosh (1969) and Robinson and Pierce (1964) have shown that it is a glycoprotein containing fucose, mannose, galactose and seven sialic acid residues. They have also shown that human bone, liver and kidney alkaline phosphatases contain sialic acid residues but intestinal alkaline phosphatase does not. Placental alkaline phosphatase was shown by ultracentrifugation analysis to be a dimer of about 120,000 daltons with non-identical subunits of identical molecular weight (Gottlieb and Sussman, 1968). This molecular weight value for placental alkaline phosphatase is in agreement with results obtained by other workers: 116,000-125,000 (Harkness, 1968; Sussman and Gottlieb, 1969). Using

Sephadex G-200 gel filtration Moss (1970) reported higher molecular weights for human alkaline phosphatases of 225,000 for liver, 190,000 for intestinal, 180,000 for bone, and 170,000 for kidney.

(c) Sialic Acid Composition and the Isoelectric Points of the Alkaline Phosphatase Isoenzymes

Sialic acid is composed of neuraminic acid substituted at the amino residue by an acyl group. It is found predominantly in oligosaccharides and glycoproteins. Ghosh (1969) has shown that the isoenzymes of alkaline phosphatase except the intestinal one contain sialic acid residues as outlined in I.D.1.(b) above. However, little evidence has been found as to the difference in the number of sialic acid residues on the isoenzymes. Usategui-Gomez and Tarbutton (1974) suggested that sialic acid may be the determinant of the electrophoretic mobility seen between the isoenzymes. The isoelectric points (pH_I's) of several of the isoenzymes have been determined. Usategui-Gomez, Yeager and Tarbutton (1974) determined the pH_I of the placental isoenzyme to be 4.6, human liver to be 3.9 and calf intestine to be 4.4. They used isoelectric focusing to make their determination. Latner, Parsons and Skillen (1970) using a sucrose density technique obtained a pH_I of human liver to be 3.9. Greene and Sussman (1973) using isoelectric focusing determined

the pH_I of human liver to be 3.8 and that of placental to be 4.45.

(d) Kinetic Properties of Alkaline Phosphatase

There are many tissue specific differences among the alkaline phosphatases that can be used to identify the tissue origins of the serum enzymes as an aid in diagnosis. Liver and bone isoenzymes are not inhibited by 5 mM L-phenylalanine (Rhone and Mizuno, 1972; Winkleman et al, 1972) and are unstable in 3 M urea (Horne, Cornish and Posen, 1968; Birkett et al, 1967), while intestinal and placental are not inhibited by L-phenylalanine but are more stable in urea.

Michaelis constants of 6.0×10^{-5} M and 8.0×10^{-4} M at pH 9.0 and 10.5 respectively were obtained for p-nitrophenyl phosphate using human liver alkaline phosphatase (Eaton and Moss, 1968). A K_m of 8.0×10^{-4}M p-nitrophenyl phosphate at pH 10.5 was obtained for placental alkaline phosphatase by Harkness (1968) and a K_m of 8.3×10^{-4}M p-nitrophenyl phosphate was obtained for placental alkaline phosphatase by Usategui-Gomez, Yeager and Tarbutton (1974). Calf scapula cartilage alkaline phosphatase was found to have a K_m of 5.9×10^{-4}M for p-nitrophenyl phosphate at pH 10.0 and 37° (Vittur and deBernard, 1973).

(e) Metal Ion Effects on Alkaline Phosphatase

Alkaline phosphatase requires metal ions for

activity and dialysis against 0.01 M KCN or EDTA for 6 days produces an inactive calf intestinal enzyme (Hofstee, 1955). Incubation of dialyzed pig kidney alkaline phosphatase with Mg^{++} gave 40% recovery and incubation with Zn^{++} gave 70% recovery of activity to the enzyme. Harkness (1968) did much to clarify the situation of metal ion requirements. Working with Zn^{++} chelating agents he showed that they gave almost 100% inhibition of activity. Addition of 100 mM Zn^{++} immediately restored full activity compared to only 25% restoration of activity when Mg^{++} was added. He thus showed that Zn^{++} is an essential metal ion for alkaline phosphatase activity and that Mg^{++} is much less effective. However, activation of various tissues phosphatases has been observed with Ca^{++}, Mg^{++}, and Mn^{++} (Morton, 1955; Ahmed and King, 1960); while other ions, notably Be^{++}, and Zn^{++} at high concentrations are inhibitory. Few details are available on the kinetics of Zn^{++} inhibition, which is surprising in view of the absolute requirement for this ion. One report (Moss, 1969) states that it is non-competitive or mixed inhibition. Mg^{++}, in the presence of optimum Zn^{++}, does not affect the affinity for the substrate and it may act by increasing the number of active sites rather than by enhancing a particular rate at one site (Fernley and Walker, 1970).

2. Electrophoretic Properties of Alkaline Phosphatase

(a) Electrophoresis

Electrophoresis provides a useful technique for separating the isoenzymes of alkaline phosphatase on the basis of their different electrical charges. Much work has been done employing cellulose acetate (Rhone and Mizuno, 1972; Fritsche and Adams-Park, 1972); agarose (Winkleman et al, 1972), agar gel (Yong, 1967); although starch (Rufo and Fishman, 1972; Hill and Sammons, 1967) and polyacrylamide (Smith et al, 1968) gels produce the best resolution of the proteins.

(i) Cellulose Acetate and Agarose

On cellulose acetate medium when compared with normal serum proteins (Rhone and Mizuno, 1972; Rhone, White and Gidaspow, 1973), liver alkaline phosphatase migrates in the α_2 region. Some specimens may contain a "fast" liver band which migrates in the α_1 region. Bone migrates just behind the liver band in the β-region and forms a band which is usually diffuse and cannot always be distinguished from the liver band. Intestinal alkaline phosphatase migrates considerably behind the liver and bone bands having a major band in the γ-globulin region. The placental isoenzyme migrates between the bone and the intestinal isoenzymes.

Winkleman et al (1972) and Ewen (1974) were able

obtain essentially the same quality of separation of the isoenzymes of alkaline phosphatase using agarose gels as could be obtained using cellulose acetate medium. However, it was found that isoenzyme patterns were seen in diseases in which the tissue of origin of the isoenzyme was not primarily involved in the disease, particularly in diseases involving kidney, or intestine. However when the bone or liver was implicated in the disease process, the particular isoenzyme was seen in the serum making the technique valuable in distinguishing liver and bone diseases.

(ii) Polyacrylamide Gel

Recently, work has been done on separating the alkaline phosphatase isoenzymes on polyacrylamide gels. Smith, Lightstone and Perry (1968) were able to achieve good separation on 5% acrylamide disc gels. Liver extracts showed a band running with the major band of serum. The bone extracts showed a slower, slightly more diffuse band distinguishable from the liver band. These results are supported by Walker and Pollard (1972) working with polyacrylamide slabs. They demonstrated the presence of a 'fast' liver band in liver tissue extracts and sera from patients with hepatobiliary diseases that was not in normal sera. The 'fast' liver band was not seen in work done by Dingjan, Postma and Stroes (1973) who were only able to detect one liver band, bone and intestinal bands

clearly separable from each other on disc gel electrophoresis.

(b) Isoelectric Focusing

Isoelectric focusing can be used for the quantitative evaluation of the isoenzymes as well as for preparative purposes outlined in I.C.3. Isoelectric focusing has recently been shown to be a powerful tool for separation of proteins (Vesterberg and Svenson, 1966) and a few workers have applied this technique to separation of alkaline phosphatase isoenzymes. Smith, Lightstone and Perry (1971) used pH 3-10 ampholine in their gels which were 5.5% total polyacrylamide. They determined no isoelectric points but found the human alkaline phosphatases to focus in the pH range 4-6. Clear, distinguishable bands were obtained for each of the serum isoenzymes. More recently workers have obtained more concise isoelectric points (pH_I's): pH_I 4.3-4.6 for baboon liver (Hammond et al, 1973), pH_I 3.9 for human liver (Usategui-Gomez et al, 1974), pH_I 3.8 for human liver (Greene and Sussman, 1973).

3. Effects of Miscellaneous Treatments on Alkaline Phosphatase

(a) Neuraminidase Treatments

All the human tissue isoenzymes of alkaline phosphatase except the intestinal isoenzyme contain sialic acid residues as outlined in I.D.1.(b) above. Robinson and Pierce (1964) have shown that incubation of

the isoenzymes from liver, bone and placenta with neuraminidase slows their electrophoretic migration. They postulated that this was due to the removal of the terminal sialic acid residues which contribute to the net negative charge on the enzyme. Butterworth and Moss (1970) showed that phosphatases derived from one tissue shown to be heterogeneous on electrophoresis could be reduced to one compact zone of activity if they were incubated with neuraminidase. This suggests that the difference in the isoenzymes within a tissue is due to their sialic acid composition. Most data suggest that within a single tissue there is only one type of alkaline phosphatase and that the observed heterogeneity results from modification of a single enzyme to give a population of molecules with varying net electrical charge but similar size and enzyme activity (Moss, 1970). Treatment of alkaline phosphatase with neuraminidase does not effect the enzymatic activity (Smith, Lightstone and Perry, 1968; Ghosh and Usategui-Gomez, 1969).

(b) Triton X-100 Treatments

Alkaline phosphatase forms aggregates as outlined in I.D.4 below that, on electrophoresis do not appear to enter 5% polyacrylamide gels. Fishman (1974) showed that regardless of the individual source of the alkaline phosphatase, 0.5% Triton X-100, when added to the gels

and samples, caused more enzyme protein to enter the gel than when the detergent was absent.

(c) Heat Treatments

Posen et al (1965) did work on the heat stability of the isoenzymes of alkaline phosphatase. They found that the bone isoenzyme, relative to the liver and intestinal isoenzymes, is labile to heat denaturation (10 min at 56°). Contrarily, the placental isoenzyme, relative to the liver and intestinal isoenzymes, is more stable to heat treatment. The placental isoenzyme retains 60% of its original activity after incubation at 65° for 10 min (Ghosh, 1969), but at this higher temperature the increased loss of activity of the liver and bone isoenzymes, makes it of little use for differentiating the tissue source of these two isoenzymes.

More recently a new approach has been presented for the identification of increased levels of serum alkaline phosphatase isoenzyme activity by heat inactivation based on the patterns of enzyme stability at 56° over a 20 min period (Cadeau and Malkin, 1973). Using the assessment of relative heat stability patterns, the authors were able to differentiate between liver and bone as the tissue source of increased serum alkaline phosphatase in 93% of the cases of patients with diagnosed liver and bone disease, compared to 78% predictability of the cases using the 10 min

thermostability test. This technique eliminates the variation which may occur in the 10 min thermostability test because of variations in incubation temperature from day to day.

4. Aggregation of Alkaline Phosphatase

Recently several workers have described the phenomenon of aggregation of alkaline phosphatase molecules (Ferwerda and Stepan, 1973; Fritsche and Adams-Park, 1974). They describe a molecule which comes off the Sephadex G-200 column in the void volume. After passage through the same column in the presence of 0.1% sodium deoxycholate, an ionic detergent (Vittur and deBernard, 1973), or after treatment with butanol (Jennings, Brocklehurst and Hirst, 1970) the alkaline phosphatase is retarded on the Sephadex G-200 column. Jennings, Brocklehurst and Hirst (1970) further showed that this high molecular weight enzyme fraction is retained at the origin of starch gel support media and that the origin band disappears after treatment with butanol. It has been suggested by Newton (1967) and Dunne, Fennelly and McGeeney (1967) that the aggregate could be a lipoprotein complex which is disrupted by butanol treatment.

E. Functions of Alkaline Phosphatase Isoenzymes

1. Intestinal

The intestinal alkaline phosphatase has been

postulated to be involved in the absorption of lipid from the intestinal tract (Langerman et al, 1966; Warnock, 1968). More recently, it has also been postulated to be involved in calcium transport in the small intestine and has been considered to be the same enzyme as calcium-activated adenosine triphosphatase (EC 3.6.1.3) (Hanssler et al, 1970; Russell et al, 1972; Taylor and Wasserman, 1969; Gasser et al 1972). If alkaline phosphatase is a calcium adenosine triphosphatase, then it may be significant that it is particularly abundant in tissues such as intestine, kidney, bone and placenta, all of which play a role in calcium metabolism. Studies done on purified intestinal alkaline phosphatase using substrates associated with other specific phosphatase activities (Narayanan and Appleton, 1972b) show intestinal alkaline phosphatase to have these other enzyme activities associated with it: inorganic pyrophosphatase (EC 3.6.1.1), adenosine triphosphatase (EC 3.6.1.3), as well as D-glucose-6-phosphatase (EC 3.1.3.9), D-glucose-1-phosphatase (EC 3.1.3.10) and D-fructose-1,6-disphosphatase (EC 3.1.3.11).

2. Bone

Evidence suggests that alkaline phosphatase in bone plays some role in the calcification process in bone, but its precise function is still a matter of speculation. Ali, Sajdera and Anderson (1970) have isolated extracellular

matrix vesicles from epiphyseal cartilage and showed them to contain alkaline phosphatase, pyrophosphatase and adenosine triphosphatase activities. Vreven, Lieberherr and Vaes (1973) and Fleisch and Russell (1970) support the proposal that bone alkaline phosphatase could be identical to alkaline inorganic pyrophosphatase.

3. Liver and Bile

Little work has been done on associated phosphatase activities involved with liver alkaline phosphatase. The liver has been proposed to act in clearing alkaline phosphatase produced by the bone (Gutman, 1959) and the intestinal mucosa (LeVeen et al, 1950). It has also been shown however, that the liver synthesizes alkaline phosphatase and secretes it into the bile (Chenderovitch, 1968; Polin et al, 1962); Sebesta, Bradshaw and Prockop, 1964).

4. Placenta

The function of placental alkaline phosphatase remains to be definitely established, however, the enzyme does have inorganic pyrophosphatase activity associated with it (Horder, 1974).

F. Isoenzymes of Alkaline Phosphatase

1. Use of Alkaline phosphatase is of clinical interest because it is known to be increased in the sera of patients with certain pathological conditions. The liver isoenzyme is reported to be elevated in the sera of patients with

obstructive jaundice[1], parenchymal liver disease[2], cirrhosis[3], and toxic hepatitis[4]. The bone isoenzyme is elevated in the sera of patients with Paget's disease[5], rickets[6], healing fractures and hyperparathyroidism[7]; intestinal alkaline phosphatase has been found to be elevated in the sera of patients with ulcerative colitis[8], cirrhosis, hepatobiliary disorders and chronic renal failure; finally, increased serum levels of placental

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1. Obstructive jaundice: A syndrome characterized by hyperbilirubinemia and deposition of bile pigments in the skin and mucous membranes due to the impedment of bile flow from the liver to the duodenum.

2. Parenchymal liver disease: A disease of the parenchymal or functional element of the liver.

3. Cirrhosis: A disease of the liver marked by progressive destruction of liver cells, accompanied by regeneration of the liver substance and increase of connective tissue.

4. Toxic hepatitis: Inflammation of the liver due to the direct action of a poison on the liver cells.

5. Paget's disease: Inflammation of a bone, involving the haversian spaces, canals and their branches and generally the medullary cavity; marked by enlargement of the bone, tenderness, and a dull aching pain.

6. Rickets: A condition caused by deficiency of Vitamin D, with disturbance in normal ossification.

7. Hyperparathyroidism: Abnormally increased activity of the parathyroids, causing loss of calcium from the bones and resulting in a condition marked by pain and tenderness in the bones, spontaneous fractures, muscular weakness and abdominal cramps.

8. Ulcerative colitis: Chronic ulceration in the colon with inflammation.

alkaline phosphatase are found in the sera of pregnant women. Placental alkaline phosphatase has been proposed to be of possible use as an indicator of fetal well-being and maturity in high risk pregnancies (Usategui-Gomez, Yeager and deCastro, 1973). It remains at a high level throughout the third trimester of the pregnancy and drops to pre-pregnancy levels a short time (a few days) before term.

2. The Regan Isoenzyme

An abnormally present isoenzyme of alkaline phosphatase has been found in the sera of certain individuals with malignancies. It was first described by Fishman et al (1968) and named the Regan isoenzyme after the patient in whom it was discovered. The Regan isoenzyme is produced by the malignant tissue and has been found to be indistinguishable from the human placental isoenzyme with respect to several physical, electrophoretic and immunological factors (Fishman, 1969; Fishman, Inglis and Green, 1971). Fishman, Inglis and Green (1971) describe the Regan isoenzyme as a carcinoplacental antigen.

The fundamental significance of the existence of normal placental phenotypes in the isoenzyme of cancer tissue and serum is that one can presume that the tumor and the placental alkaline phosphatases represent the same gene products (Greene and Sussman, 1973). Synthesis

of these proteins is a result of derepression of the genome which accompanies neoplastic transformation. Regan isoenzyme has been found in approximately 12% of cancer patients, primarily in those involving carcinomas of the lung, breast, ovaries, pancreas and stomach. Its significance lies in the fact that treatment of the carcinoma can be monitored through the levels of placental-like alkaline phosphatase.

G. Escherichia coli Alkaline Phosphatase

There has been a great deal of work done on E. coli alkaline phosphatase since its preparation is a relatively simple matter due to the stability of the enzyme, its cellular location and the ease with which constitutive mutants can be obtained resulting in the preparation of gram quantities of the enzyme. Under phosphate limiting conditions, 6% of the total protein produced by wild-type E. coli strain can be alkaline phosphatase (Garen and Levinthal, 1960). E. coli alkaline phosphatase is a metalloenzyme reputed to contain 4 zinc atoms (Simpson and Vallee, 1968; Lazdunski et al, 1969) per dimer molecular weight (Rothman and Byrne, 1963). Its molecular weight was found to be 86,000 (Garen and Levinthal, 1960). There are two active sites per dimer molecule, but only one of them is reactive at a given time (Halford, 1970).

Neu and Heppel (1965) suggested that E. coli alkaline phosphatase is located between the outer cell wall and the plasma membrane. The repression of alkaline phosphatase biosynthesis by inorganic phosphate and the relationship between cell growth rate and alkaline phosphatase content when β-glycerophosphate is the only carbon source, suggest that the physiological role of the enzyme in E. coli is the provision of inorganic phosphate (Halford, 1970). Removal of zinc at neutral pH by dialysis against a chelator causes the loss of all enzymatic activity but only minor changes in protein structure (Reynolds and Schlesinger, 1969). Readdition of zinc to the apo-enzyme reactivates it. Magnesium can also substitute for zinc to obtain a partially active enzyme (Schlesinger, 1966). Also, as the pH is lowered to pH 2 the dimer will dissociate to a monomer which exists as an extended coil with no enzymatic activity. Restoration of the pH restores the monomer and reassociates the active dimer (Reynolds and Schlesinger, 1967).

A mechanism for the hydrolysis of phosphate monoester (ROP) by E. coli alkaline phosphatase has been proposed by Wilson, Dayan and Cyr (1964) and supported by Halford (1971):

$$E + ROP \rightleftharpoons E.ROP \rightleftharpoons E.Pi + ROH \rightleftharpoons E + Pi$$

in which the rate limiting step is always the dephosphoryl-

ation of the enzyme. ROH is the alcohol released on hydrolysis of the ester ROP; E.ROP and E.Pi are enzyme-substrate and -orthophosphate complexes. Halford (1970) suggested that the provision of a better phosphatase acceptor than water accelerates the dephosphorylation rate and this causes an increase in k_{cat} when dephosphorylation is rate limiting.

More recently isoelectric focusing of E. coli alkaline phosphatase gave 5 isoenzymes in the pH range 5.06 - 5.39 (Csopak, Jonsson and Hallberg, 1972). They also found that were was a loss of alkaline phosphatase activity during the electrolysis procedure that could be restored by the addition of zinc.

CHAPTER II

MATERIALS AND METHODS

A. Source of Materials

1. Chemical Compounds

Routine chemicals (reagent grade) were from Fisher Scientific Co. Ltd., Eastman Organic Chemicals, or J. T. Baker Chemical Co. Biochemicals were generally of the highest purity available and were from the following sources: p-nitrophenyl phosphate, 2-amino-2-methyl-1-propanol (AMP),rabbit muscle phosphorylase a, naphthol AS-MX phosphoric acid, rabbit muscle aldolase, Escherichia coli alkaline phosphatase, β-naphthol phosphate, phenolphthalein monophosphate, β-glycerophosphate from Sigma Chemical Co.; acrylamide from Eastman Kodak Co.; TEMED and Coomassie Blue R-250 from Bio-rad Laboratories, Inc.; ampholine from LKB Instruments, Inc.; neuraminidase and human serum albumin from Calbiochem; bovine liver glutamate dehydrogenase from Boehringer Mannheim; egg white ovalbumin from Worthington; rabbit muscle glyceraldehyde-3-phosphate dehydrogenase prepared by the method of Ferdinand (1964); pig muscle lactate dehydrogenase prepared by Dr. R. A. Stinson (Stinson and Gutfreund, 1971); rabbit muscle pyruvate kinase gift of Dr. J. J. Holbrook, University of Bristol; yeast phosphoglycerate kinase prepared by Dr. R. A. Stinson (Stinson, 1974); DEAE-cellulose ion exchange

resin (Whatman DE52) from W. and R. Balston, Ltd.; concanavalin A-Sepharose and Sephadex G-200 from Pharmacia. All water used was glass distilled and deionized and contained 2 ppm ionizable impurities.

2. Instrumental Equipment

The electrofocusing apparatus was from Medical Research Apparatus, Inc.; disc electrophoresis and DC power supply Model No. 3-1014A from Buchler Instruments; Technicon AA proportioning pump from Technicon Instruments Corp.; LKB Ultrorac fraction collector from LKB-Produkter; pH meter-type pH M26 from Radiometer Corp. equipped with a microprobe combination electrode from Fisher Scientific Co.; Unicam SP800 thermoregulated spectrophotometer with Unicam SP21 recorder and scale expansion accessories; Polaroid MP-3 camera with Polaroid black and white film-land type 107 from Polaroid Corp.; Blak-ray long wave ultraviolet lamp from Ultra-Violet Prod. Inc.; fluorescent light box from Shandon Sci. Co. Ltd.

B. Purification of Tissue Alkaline Phosphatase

1. Preparation of the Tissue for Extraction

All tissues used were obtained within twelve hours of death from autopsy specimens, and if not used immediately were frozen at -70°. The tissues were semi-thawed before use. The entire procedure was carried out in the cold room at 4° and/or on ice. A typical pre-

paration of alkaline phosphatase involved 500 g of tissue. The volumes and measurements outlined are for this size of a preparation.

(a) Liver

For the liver alkaline phosphatase purification the livers were stripped of any vessels (arteries and ducts), the outer membrane removed and the tissue sliced into roughly one inch cubes before homogenization.

(b) Bone

For the purification of bone alkaline phosphatase, ribs from autopsy specimens were obtained. The ribs were cut in approximately six inch segments, cleaned of connective tissue and muscle and were deep frozen (-70°). They were then broken into small fragments either by crushing or with a saw. These fragments were again frozen and ground through a meat grinder which had been precooled in the freezer. The resulting chips were again frozen and crushed in a plastic bag with a hammer. The resultant tissue was then carried through the extraction procedure.

(c) Placenta

Placental tissue was obtained within two hours of parturition, the amniotic membrane removed and the tissue washed free of clotted blood under cold running tap water. The tissue was then blotted and cut into one inch segments and carried through the extraction procedure.

(d) Intestine

The intestine was cut open longitudinally and the mucosa was scraped from the intestinal wall with a razor blade. The resultant tissue was then carried through the extraction procedure.

2. Homogenization and Butanol Extraction

The methodology used was essentially that of Moss et al (1967). For the initial preparations of liver alkaline phosphatase, the buffer used was 10mM Tris-base, pH 7.6 (HCl). To 500 g of tissue was added 1000 ml of this buffer and the mixture was homogenized in a Waring blender (5 min at low speed and 2 min at high speed). The homogenate was then extracted with cold (4°) butanol (750 ml were added to 1500 ml of homogenate) for 30 min using an overhead stirrer. The extraction mixture was then centrifuged at 9,000 x g for 30 min in a refrigerated Sorvall centrifuge. The aqueous layer (approximately 1000 ml) was recovered. In the most recent preparations of tissue alkaline phosphatase, the butanol was cooled to -20° prior to use in an effort to limit losses due to organic solvent effects and the Tris buffer was supplemented with 0.1 mM $MgCl_2$ and 0.01 mM $ZnCl_2$ to increase enzyme stability.

3. Acetone Fractionation

The aqueous extract recovered from the butanol extraction step (1000 ml) was made 30% (v/v) acetone by

adding 430 ml cold (4°) acetone slowly while the mixture was allowed to stir slowly for 15 min and was centrifuged at 9,000 x g for 20 min. The resulting supernatant (1400 ml) containing the alkaline phosphatase was made 50% (v/v) acetone by the slow addition of 550 ml of cold acetone while the mixture was being stirred with the overhead stirrer. The mixture was then centrifuged at 9,000 x g for 20 min and the pellet containing the alkaline phosphatase activity was recovered. In the most recent preparations the acetone was cooled to -20° prior to use to stabilize the enzyme against organic solvent effects. The 30 - 50% acetone pellet was taken up in buffer, typically 350 ml of 10 mM Tris-base, pH 7.6 (HCl) containing 0.1 mM $MgCl_2$, 0.02 mM $ZnCl_2$.

4. Ammonium Sulfate Fractionation I

This procedure was carried out on ice with the aid of a magnetic stirrer. To the 30 - 50% acetone pellet taken up in 350 ml of buffer at 0° was added 110 g of solid ammonium sulfate resulting in 50% saturation (50% of 4.1 M which is saturation at 25°) with respect to the salt. The supernatant containing the alkaline phosphatase was recovered by centrifugation at 17,000 x g for 20 min. To the supernatant was added an additional 53 g ammonium sulfate to bring the solution to 70% saturation with respect to the salt. Centrifugation at 17,000 x g for 20

min yielded a pellet that was taken up in approximately 50 ml of 10 mM Tris-base buffer pH 7.6 (HCl) containing 0.1 mM $MgCl_2$, 0.02 mM $ZnCl_2$ and dialyzed against 1000 ml of the same buffer overnight at 4°. The buffer was changed three times during the dialysis. This dialyzed alkaline phosphatase solution, approximately 80 ml, was then ready for further chromatographic purification or it could be stored in this state.

5. DEAE-cellulose Ion Exchange Chromatography

DEAE-cellulose (125 g of resin) was treated with 250 ml of 0.1 N HCl, dried in a Buchner funnel under vacuum, washed with three 500 ml aliquots of deionized water drying the resin between each wash. The DEAE-cellulose was then treated with 250 ml of 0.1 N NaOH, dried, washed with three 500 ml aliquots of deionized water and washed with 10 mM Tris-base buffer, pH 7.6 (HCl) containing 0.1 mM $MgCl_2$, 0.02 mM $ZnCl_2$ (column buffer). The pH was adjusted to pH 7.6 if necessary and the resin was washed a second time with the column buffer. The column (3 cm diameter x 15 cm height) was packed under gravity; the column was sealed and the column buffer was pumped through for 6 hr to equilibrate the column. The flow rate was 120 ml per hr. The sample, 80 ml of the 50 - 70% ammonium sulfate fractionated sample containing 1.5 g of protein was pumped onto the column and the column washed

with 50 ml of column buffer to elute the unbound proteins. Elution of the alkaline phosphatase was accomplished with a linear NaCl gradient generated with 500 ml of column buffer in the mixing flask and 500 ml of column buffer plus 0.2 M NaCl in the reservoir flask. Fractions of 3 ml were collected and the tubes that contained significant alkaline phosphatase activity were pooled.

6. Concanavalin A-Sepharose Affinity Chromatography

Concanavalin A covalently bound to Sepharose 4B (Con A-Sepharose) was used to separate alkaline phosphatase by affinity chromatography. Initially the column buffer was 100 mM Tris-base, pH 7.6 (HCl) plus 10 mM $CaCl_2$, 10 mM $MnCl_2$. The $CaCl_2$ and $MnCl_2$ were added to the buffer to maintain the binding properties of concanavalin A. The column was 1 cm diameter x 15 cm height and was run under gravity with a flow rate of 48 ml per hr. A 50 mM glucose solution in the column buffer was tried initially for use in washing loosely bound glycoproteins from the column but was found to elute significant amounts of alkaline phosphatase activity and hence was abandoned in favor of a simple buffer wash after sample application. The pH of the column buffer was lowered to pH 7.2 (100 mM N-Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer, pH 7.2 (HCl) plus 10 mM $CaCl_2$, 10 mM $MnCl_2$) to achieve stronger binding of alkaline phosphatase by con-

canavalin A. However, it was found that 100 mM methyl-α-D-glucopyrannoside in the column buffer and 100 mM methyl-α-D-mannoside in the column buffer were required to elute the alkaline phosphatase activity and that this activity was eluted in a broad trailing peak. The column buffer finally established for future use was 100 mM Tris-base, pH 7.6 (HCl) plus 100 mM NaCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$ and the alkaline phosphatase was eluted with 100 mM mannose in the column buffer.

For the most recent liver alkaline phosphatase preparations 20 ml of Con A-Sepharose (packed resin) was equilibrated by successive column buffer washes and Buchner funnel drying until the pH of the slurry was constant at 7.6. The slurry was poured into a 1 cm diameter glass column under gravity to a height of 25 cm. The column was run under gravity with a flow rate of 48 ml per hr. The packed column was washed with 200 ml of the column buffer and the pool from the DEAE-cellulose column was applied in 100 ml. The column was washed with 50 ml of the column buffer to elute the unbound proteins (presumably non-glycoproteins) and the alkaline phosphatase was recovered with a further wash of 100 mM mannose in the column buffer. The fractions from this step elution were 8.0 ml. The column was regenerated by passing through 100 ml of the column buffer adjusted to pH 9.5 with NaOH followed by

50 ml of the original column buffer pH 7.6. The regenerated column was sealed and stored for future use at 4°.

7. Sephadex G-200 Gel Permeation Chromatography

Sephadex G-200 was used to further purify alkaline phosphatase on the basis of its size and molecular weight. Sephadex G-200 was packed under gravity into a 2.5 cm diameter x 90 cm long column. Initially, equilibration was with 10 mM Tris-base buffer, pH 7.6 (HCl) but in the most recent preparations the buffer was changed to 100 mM Tris-base, pH 7.6 (HCl) plus 100 mM NaCl, 0.1 mM $MgCl_2$, 0.02 mM $ZnCl_2$ to reduce non-specific adsorption of proteins to the Sephadex matrix. At least 1 litre of buffer was pumped onto the column by upward flow with a peristaltic pump; the flow rate was 18 ml per hr and 4.5 ml fractions were collected. The sample was applied in a volume of 20 ml or less and the alkaline phosphatase was eluted with the Tris, NaCl, $MgCl_2$, $ZnCl_2$ column buffer. Because the volume of the pool from the Con A-Sepharose column was often almost 40 ml, this sample was split and the G-200 column run twice. The high activity fractions were pooled and fractionated with ammonium sulfate.

8. Ammonium Sulfate Fractionation

The pool (84 ml) from the G-200 column was further purified by fractionation with ammonium sulfate at 4°. The sample was dialyzed overnight at 4° against 1 litre

of 50% (4.1 M is saturation at 25) ammonium sulfate in 100 mM Tris-base buffer, pH 7.6 (HCl) plus 100 mM NaCl, 0.1 mM $MgCl_2$, 0.02 mM $ZnCl_2$ with one change of buffer. The 38 ml of dialysate obtained was centrifuged at 30,000 x g for 30 min. The supernatant which contained the alkaline phosphatase activity was then dialyzed against 1 litre of identically buffered 65% ammonium sulfate overnight with one change of buffer. The 31 ml of dialysate obtained was centrifuged at 30,000 x g for 30 min. The supernatant containing the alkaline phosphatase activity was finally dialyzed against 1 litre of buffered 80% ammonium sulfate overnight with one buffer change. The 18 ml of dialysate obtained was centrifuged at 30,000 x g for 30 min. The pellet containing the alkaline phosphatase activity was brought up in the same Tris, NaCl, $MgCl_2$, $ZnCl_2$ buffer that contained 10% glycerol and stored at 4°.

C. Protein Determination

The protein content of the purified alkaline phosphatase fractions and other samples was determined by the method of Lowry et al (1951). Human serum albumin was used as a standard. The samples were made up in 200 µl volumes to which was added 1.5 ml copper sulfate-potassium tartrate reagent. Fifty µl phenol reagent was added and after 30 min the absorbance was measured with a Beckman DU spectrophotometer at 750 nm.

D. Enzymatic Assay for Alkaline Phosphatase

The method used to assay alkaline phosphatase was that of Bowers and McComb (1966). The assay medium contained 10 mM p-nitrophenyl phosphate, 1.5 mM $MgCl_2$ in 0.782 M 2-amino-2-methyl-1-propanol (AMP) buffer, pH 10.3 (HCl). The sample (up to 25 µl) was added to 1 ml of assay medium in a 1 cm path length cuvette and the rate of the reaction was continuously recorded at 404 nm at 30° with a Unicam SP800 spectrophotometer with recorder and scale expansion accessories. The absorbtivity at 404 nm of a 1 mM solution of p-nitrophenol, the product formed, is 16.7 (Halford, 1970). The activity was calculated in international units where 1 unit (U) is that amount of enzyme that will hydrolyze 1 µmole of p-nitrophenyl phosphate per min at 30°.

E. Isoelectric Focusing of Alkaline Phosphatase on Polyacrylamide Gels

Isoelectric focusing is a technique that separates proteins in a pH gradient on the basis of their isoelectric points (Righetti and Drysdale, 1971). This technique has been applied to alkaline phosphatase.

1. Gel Preparation and Composition of Buffer Solutions

The gel mixture contained 4% (w/v) acrylamide, 0.02% (v/v) TEMED, 2% (w/v) ampholine, 10% (v/v) glycerol, and 0.25 mM $ZnCl_2$ and 0.05% (w/v) ammonium persulfate as a

polymerization catalyst. For the separation of serum and tissue isoenzymes of alkaline phosphatase a 4% polyacrylamide gel that contained 1% pH 4-6 ampholine and 1% pH 3-10 ampholine was used. For the separation of neuraminidase treated samples a 4% polyacrylamide gel that contained 1% pH 5-8 ampholine and 1% pH 7-9 ampholine was used. The ammonium persulfate was the last ingredient to be added to the gel mixture. Following this addition the gel mixture was degassed and pipetted to a height of 9.3 cm in 3 mm I.D. x 10.5 cm long plastic tubes which had been sealed with parafilm on the bottom. The gels were polymerized under 15 µl of water carefully layered above the gel mixture with a syringe.

The upper or cathodal buffer was 0.02 M NaOH plus 0.25 mM $ZnCl_2$ and the lower or anodal buffer was 0.01 M H_3PO_4 plus 0.25 mM $ZnCl_2$. These buffers were precooled to 4° before use.

2. Electrofocusing Apparatus

The focusing was carried out in a Medical Research Apparatus focusing system. The gels were positioned vertically between the two buffer chambers and were cooled to -0.5° by a circulating water-methanol bath.

3. Gel Preparation and Running Procedure

The parafilm was removed from the bottom of the gel tubes and a piece of towelling wick held in place with

a rubber sleeve was substituted. The gels were put into the electrofocusing apparatus which had been precooled to -0.5°. The precooled buffers were put into the appropriate chambers, the air removed from the tops of the gels by squirting buffer onto the gel surface and the gels were prefocused without the samples at 0.5 mA per gel (constant current) until the voltage reached 400 V and then this voltage was maintained (constant voltage) until a total prefocusing time of 1 hr was obtained. The prefocusing discharged the excess ammonium persulfate from the gel and partially forms the pH gradient.

The samples (0.003 U of alkaline phosphatase activity if the gels were to be stained for enzyme activity or 5 µg of protein if the gels were to be stained for protein) were mixed 1:1 with a 50% sucrose solution which contained 8% pH 6-8 ampholine. The sample volume before sucrose addition was never larger than 50 µl. The samples were layered on the prefocused gels under a layer of 15 µl of 20% sucrose solution which contained 2% pH 6-8 ampholine. The samples were then focused 16-18 hr at 400V.

4. Removal of the Gels from the Running Tubes

When focusing was completed the tubes were removed from the apparatus and the gels were extruded by gently forcing them out into the hand with a pasteur pipette bulb. The gel was washed with 1 ml of 0.02 M AMP,

pH 9.8 (HCl) and the bottom was marked with India ink. The gels were stained for either alkaline phosphatase activity or protein as outlined under section II.H below.

F. Polyacrylamide Gel Electrophoresis

The method used for disc electrophoresis was as described by Dietz, Lubrano and Rubenstein (1971). The gels were composed of 5.5% (w/v) acrylamide, 0.15% (w/v) N,N-methylene bisacrylamide, 0.02% (v/v) TEMED, 275 mM Tris-base, pH 8.9 (HCl) and polymerized with ammonium persulfate at a final concentration in the gel mixture of 0.05% (w/v). The ammonium persulfate was the last ingredient to be added to the gel mixture and following this addition the gel mixture was degassed and pipetted to a height of 6.5 cm in glass tubes 0.5 cm I.D. x 7.5 cm long which had been stoppered with small vacutainer stoppers. The gel was allowed to polymerize for 1 hr under a layer of 25 µl of water carefully layered on top of the gel mixture with a syringe. The gels were placed in the Buchler electrophoresis assembly and the stoppers removed from the bottoms of the gel tubes. The running buffer was 5.5 mM Tris-base, 38 mM glycine, pH 8.3 precooled to 4°. The upper or cathodal chamber buffer contained 0.1% bromophenol blue which migrated on electrophoresis to mark the leading ion front of electrophoretic migration. The samples (0.003 U of alkaline phosphatase activity if the gels

were to be stained for enzyme activity or 5 µg of protein if the gels were to be stained for protein) were diluted 1:1 with 40% sucrose and applied directly to the top of the gel. The samples were electrophoresed at 5.0 mA per gel until the bromophenol blue migrated to the bottom of the gel (normally 1-1½ hr). The gels were removed from the electrophoresis chamber, placed in an ice bath and were removed from the glass tubes by rimming with a jet of water from a syringe. They were then appropriately stained.

G. Molecular Weight Determination by Sodium Dodecyl Sulfate Gel Electrophoresis

Sodium dodecyl sulfate (SDS) gels were run to determine the minimum molecular weight of alkaline phosphatase according to the method outlined by Hedges (1973). Electrophoresis was carried out in gels with different polyacrylamide concentrations, ranging from 6.5% (w/v) to 10.5% (w/v). The other ingredients were kept constant were as follows: 0.15% (v/v) TEMED, 0.025% (w/v) SDS, 25 mM sodium dihydrogen phosphate, pH 7.1 (NaOH). The gels were polymerized with ammonium persulfate at a concentration of 0.05% (w/v) in the gel mixture. The gel mixture was degassed and pipetted to a height of 6.5 cm in glass tubes 0.5 cm I.D. x 7.5 cm long which were stoppered with small vacutainer stoppers. The gels were allowed to polymerize for 1 hr under 25 µl of water carefully layered with a syringe on top of the gel mixture. The gels were placed

in a Buchler disc electrophoresis assembly and the stopper removed from the bottom of each gel tube. The running buffer was 50 mM sodium dihydrogen phosphate, pH 7.1 (NaOH) plus 0.05% (w/v) SDS precooled to 4°. The sample proteins were incubated 2 hr at 37° with an incubation buffer that contained 100 mM sodium dihydrogen phosphate, pH 7.1 (NaOH), 1.0% (w/v) SDS, 1.0% (v/v) β-mercaptoethanol. This treatment dissociated the proteins into their molecular subunits and gave the individual polypeptide chains a rodlike configuration such that their length was proportional to their molecular weight (Segrest and Jackson, 1972). The sample, 10 µl in volume, was mixed with 50 µl of the sample application buffer that contained 25 mM sodium dihydrogen phosphate, pH 7.1 (NaOH), 0.25% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 50% (v/v) glycerol and 0.1% (w/v) bromophenol blue. The 60 µl samples were then layered on the gels and were electrophoresed at 8 mA per gel until the bromophenol blue migrated to the bottom of the gel (1-1½ hr). The gels were removed from the tubes as described in section II.F above and were stained with Coomassie Blue according to the method of Weber and Osborn (1969) as described in section II4.3 below. Destaining was accomplished in 20% (w/v) trichloroacetic acid in contrast to that used by Weber and Osborn (1969).

The mobilities of the proteins were calculated using the formula of Weber and Osborn:

$$\text{mobility} = \frac{\text{distance of protein migration}}{\text{distance of dye migration}} \times \frac{\text{length before stain}}{\text{length after stain}}$$

The mobilities (linear scale) were plotted against the known molecular weight of the standard proteins (logarithmic scale) on semi-logarithmic paper and the subunit molecular weight of the liver alkaline phosphatase was determined. The proteins used as standards and their molecular weights were as follows: rabbit muscle phosphorylase a - 92,500; human serum albumin - 68,000; bovine liver glutamate dehydrogenase - 57,500; egg white ovalbumin - 43,000; rabbit muscle glyceraldehyde-3-phosphate dehydrogenase - 37,000; and pig muscle lactate dehydrogenase - 35,000.

H. Polyacrylamide Gel Staining

1. Staining for Alkaline Phosphatase Activity

Naphthol AS-MX phosphoric acid was used to stain the gels for alkaline phosphatase activity. The stain was made up just prior to use and contained 0.006 mM naphthol AS-MX phosphoric acid, 6 mM $MgCl_2$, 0.10 mM $ZnCl_2$, 0.1% (w/v) sucrose in 1.8 M AMP buffer, pH 9.8 (HCl). The gels were removed from the electrophoresis or electrofocusing apparatus as described in II.E 4 and II.F above and stained

with 0.85 ml of the stain solution for 10-15 min in specially constructed glass tubes designed such that the small volume of staining solution was in contact with most of the gel. The stain was removed from the staining tubes and the gels were allowed to develop fluorescence until the appropriate intensity appeared.

To photograph the gels they were placed in the stain tubes on a dull black background and illuminated with two 20 inch long-wave ultraviolet lamps, one on either side of the gels. The room lights were turned off. The gels were photographed through a composite green (No. 50) Klett filter with a Polaroid MP-3 camera loaded with land pack type 107 film. The exposure time was 5-20 sec depending on the intensity of the fluorescent bands and the f-stop was 4.5.

2. Staining for Protein

The protein gels were partially removed from their running tubes as described in section II.E.4 and II.F above and the lower 5 mm of each gel was marked with India ink to permanently identify the anodal end. The excess ink was washed off with water and the gels were fixed for 10 min in 12.5% (w/v) trichloroacetic acid. They were rinsed twice with 65° water and placed in a staining solution of the following composition: 0.09% (w/v) Coomassie Blue R-250, 43% (w/v) ethanol, 10% (v/v)

acetic acid. The gels were stained at 65° for 15 min and then transferred to a destaining solution composed of 25% (v/v) ethanol, 10% (v/v) acetic acid and 65% (v/v) water. The destaining was carried out in 16 mm x 100 mm test tubes with continuous mixing on a rotator. The destaining solution was changed 3-4 times over a period of 6 hr until a light blue background was apparent. The gels were then placed in 65° 10% (v/v) acetic acid for 30 min to complete the destaining procedure. They were stored in 7% (v/v) acetic acid.

To photograph the gels, they were placed in grooves cut into a transparent white plastic slab. The slab was placed on a light box such that the surface of the slab was 2-3 inches above two 8 inch fluorescent lamps. The gels were photographed with a Polaroid MP-3 camera set at f-stop 11, 1/15 sec exposure time and fitted with a green (No. 56) Klett filter. The film used was Polaroid land pack type 107.

3. Staining for Protein on Sodium Dodecyl Sulfate Gels

The gels run for minimum molecular weight determination using SDS as described in section II.G above were stained according to the method of Weber and Osborn (1969), and destained using 20% (w/v) trichloroacetic acid. Once the gel was removed from the running tube as described in section II.F above, the bromophenol blue band was marked

with India ink injected into the gel with a syringe. The gel was placed in the following staining solution: 0.25% (w/v) Coomassie Blue R-250, 10% (v/v) acetic acid, 45% (v/v) methanol, 45% (v/v) water. They were stained in 16 mm x 100 mm test tubes mixing on a rotator for 1 hr. Twenty per cent (w/v) trichloroacetic acid was used to destain the gels since it was found to be faster than the destain of Weber and Osborn (1969). The gels were destained overnight with 4-5 changes. Once the background was clear the gels were transferred to 10% (v/v) acetic acid to change the color of the protein bands to a lighter blue. They were photographed as described in section II.4.2 above and stored in 7% (v/v) acetic acid.

4. Staining for Glycoprotein with Periodic Acid Schiff Reagent

Once the gels were removed from the running tubes as described in II.F above, the bottom of the gel was marked with India ink, the excess ink washed off and the gels were fixed for 1 hr in 12.5% (w/v) trichloroacetic acid, rinsed with water and stained by the periodic acid Schiff method of Segrest and Jackson (1972). This method involved the oxidation of the glycoprotein with periodate, reduction of excess periodate with metabisulfite and staining the oxidized glycoprotein with the Schiff reagent. After the gels were fixed for 1 hr, they were treated for 2½ hr with 0.7% (w/v) periodic acid in 5% (v/v) acetic acid.

2½ hr with 0.2% (w/v) sodium metabisulfite in 5% (v/v) acetic acid and stained with Schiff reagent (10 g fuchsin per litre water plus 200 ml 1 N HCl and 17 g sodium metabisulfite). Color development took 18 hr at room temperature. The gels were stored in 7% (v/v) acetic acid.

I. pH Gradients

The gels used for isoelectric focusing contain ampholytes which set up a pH gradient throughout the length of the gel. Gradients of pH were determined on these gels to establish the pH range that was involved in the length of the gel, to determine the linearity of the gradient and to determine the isoelectric points of the focused alkaline phosphatase isoenzymes. Gradients were done on both the 4% (w/v) polyacrylamide, 1% (w/v) pH 4-6 ampholine, 1% (w/v) pH 3-10 ampholine gels used for focusing alkaline phosphatase tissue and serum isoenzymes and on the 4% (w/v) polyacrylamide, 1% (w/v) pH 5-8 ampholine, 1% (w/v) pH 7-9 ampholine gels used for focusing the neuraminidase treated samples. The gels used to determine the pH gradients were run with a specimen applied in the manner described under section II.E above. Hemoglobin was run with the neuraminidase treated samples as a marker because it has known pH_I values - Hemoglobin A pH 7.0 and Hemoglobin A_2 pH 7.3. The gel focused for a pH gradient determination was

removed from the gel tube and cut into 3 mm segments with a razor blade. The average length of the gels was 7.8 cm resulting in 26 segments. Each segment was put in a small test tube (10 mm x 75 mm) which contained ½ ml of deionized, degassed water. The segments were allowed to sit for 1 hr to equilibrate and the pH of each segment was determined within 2 hr. This procedure should minimize the effect on pH of CO_2 dissolving in the water. The readings were done on the pH-Meter, type PH M26 equipped with a microprobe combination electrode. The remaining gels run with the gradient gel were appropriately stained for alkaline phosphatase activity, the distances of the bands measured and the R_f's of the bands determined. The isoelectric points of these bands could be obtained from the determined gradient.

J. Sephadex G-200 Gel Chromatography for Molecular Weight Determination of Alkaline Phosphatase

Sephadex G-200, particle size 40-120, will separate peptides and globular proteins in the molecular weight range 5,000-800,000 daltons[9]. Determann (1968) described experiments used to determine the molecular weights of proteins and demonstrated that the molecular weight of a protein is a logarithmic function of its elution volume.

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9. According to statements by the manufacturer: Pharmacia Fine Chemicals, Uppsala, Sweden.

For the determination of the molecular weight of alkaline phosphatase a 2.5 cm diameter x 90 cm long column of G-200 was used. The buffer was 100 mM Tris-base, pH 7.6 (HCl) plus 100 mM NaCl, 0.1 mM $MgCl_2$. The standard proteins used as molecular weight markers were: rabbit muscle pyruvate kinase - 237,000; rabbit muscle aldolase - 160,000; E. coli alkaline phosphatase - 86,000; and yeast phosphoglycerate kinase - 47,000. The prepared enzyme markers used were essentially pure as judged by the peaks obtained from the G-200 column. With the exception of aldolase (chromatographed 3 times) each of the molecular weight markers and the purified liver alkaline phosphatase were chromatographed six times. The sample size was 1 ml and 4.5 ml fractions were collected. The peaks of the E. coli and human liver alkaline phosphatase were determined by assaying the fractions for enzyme activity as described in section II.D above. The peak of pyruvate kinase was determined by monitoring the OD_{225} of the fractions. The peaks of aldolase and phosphoglycerate kinase were determined by catalytic activities (Boehringer Method; Stinson, 1974). The elution volumes of these proteins were determined by the weight of the fractions, obtained by weighing the racks of collecting tubes before and after chromatography (assuming the density of the buffer to be 1 g/ml). On semi-logarithmic paper the molecular weights

(log scale) of the standard proteins were plotted against their elution volumes (linear scale) and the apparent molecular weight of alkaline phosphatase was determined.

K. Stability Studies of Alkaline Phosphatase under Various Buffer Conditions

A portion of purified liver alkaline phosphatase was stored under each of the following conditions:

1. 10 mM Tris-base, pH 7.6 (HCl) plus 10% (v/v) glycerol at 4°
2. 100 mM Tris-base, pH 7.6 (HCl) plus 10% glycerol at 4°
3. 10 mM Tris-base, pH 7.6 (HCl) plus 50% glycerol at 4°
4. 10 mM Tris-base, pH 7.6 (HCl) at -20°
5. 10 mM Tris-base, pH 7.6 (HCl) plus 80% (w/v) ammonium sulfate at 4°

The purified liver alkaline phosphatase was assayed at 113,000 U/l and 1:50 dilutions were made into the various buffer solutions so that the initial activities would be the same value. The initial activity was 2,210 U/l. The samples were assayed at various times over a five month period and the most suitable conditions for the enzyme were determined.

L. Alkaline Phosphatase Substrate Specificity Studies

The four substrates, β-glycerophosphate, β-naphthol phosphate, phenolphthalein monophosphate and p-nitrophenyl phosphate were individually utilized as substrates for the isoenzymes of alkaline phosphatase from the tissues of liver, bone, intestine and placenta, a serum sample that

demonstrated predominantly liver alkaline phosphatase, and a serum sample that demonstrated predominantly bone alkaline phosphatase as judged by polyacrylamide disc gel electrophoresis. Comparisons were then made among isoenzymes with a common substrate and among substrates with a common enzyme source. All the activities were converted to international units (μmoles of substrate released/min/litre of enzyme solution). In order to compare these four substrates, the only variable was substrate and all other factors such as buffer composition (0.782 M AMP buffer, pH 10.3 (HCl) plus 1.5 mM $MgCl_2$) were kept constant. This was not the buffer used in the original methods utilizing these substrates.

1. p-Nitrophenyl Phosphate as a Substrate

The p-nitrophenyl phosphate was 10 mM in 0.782 M AMP buffer, pH 10.3 (HCl) plus 1.5 mM $MgCl_2$ (Bowers and McComb, 1966). The reaction rate method outlined in section II.D above was used. The reaction was run at 30°.

2. β-Glycerophosphate as a Substrate

The β-glycerophosphate was 23 mM (Shinowara, Jones and Reinhart, 1942) in 0.782 M AMP buffer, pH 10.3 (HCl) plus 1.5 mM $MgCl_2$. The alkaline phosphatases from the various sources were incubated individually with 1 ml of the substrate at 30° for 1 hr, the reactions were stopped with trichloracetic acid and the amount of phos-

phate released determined. This was converted to enzyme activity in international units.

3. β-Naphthol Phosphate as a Substrate

The β-naphthol phosphate was 10mM (Seligman et al, 1951) in 0.782 M AMP buffer, pH 10.3 (HCl) plus 1.5 mM $MgCl_2$. The alkaline phosphatase from various tissues was incubated with 1 ml of the substrate at 30° for 1 hr, the reaction was stopped with trichloroacetic acid and the phosphate released determined. This was converted to enzyme activity in international units.

4. Phenolphthalein Monophosphate as a Substrate

The phenolphthalein monophosphate was 2.5 mM (Babson et al, 1966) in 0.782 M AMP buffer, pH 10.3 (HCl) plus 1.5 mM $MgCl_2$. The enzyme was incubated with 1 ml of substrate for 1 hr at 30°, the reaction was stopped with 5 ml of color stabilizer (0.10 M trisodium phosphate, pH 11.2 (HCl)), and the tests were read against phenolphthalein standards to determine the enzyme activity.

M. K_m Determination of p-Nitrophenyl Phosphate

The K_m of p-nitrophenyl phosphate was determined on human liver alkaline phosphatase. The buffer used was 0.782 M AMP, pH 10.3 (HCl) plus 1.5 mM $MgCl_2$. The substrate concentrations ranged from 0.095 mM to 10mM. The assay procedure of Bowers and McComb (1966) was used

employing a reaction rate analysis at 30°. The rate of p-nitrophenol formation was monitored at 404 nm using 16.7 as the millimolar absorbtivity (Halford, 1970). A Lineweaver-Burk plot was drawn and the K_m was determined.

N. Heat Stability Treatments of the Isoenzymes of Alkaline Phosphatase

Purified liver alkaline phosphatase and the partially purified isoenzymes from bone, intestine and placenta were tested for their stability at 56.0° for exactly 10 min (Moss, Shakespeare and Thomas, 1972). Immediately after treatment the samples were placed in an ice bath and assayed within 30 min for the remaining alkaline phosphatase activity.

O. Neuraminidase Treatments of Liver and Bone Alkaline Phosphatases

The pH optimum for neuraminidase (Vibrio cholerae) is 6.5. The neuraminidase treatments were done at pH 6.0 and at 25°. This was achieved by incubating the alkaline phosphatase in 50 µl of inactivated serum (heat inactivated - 1 hr at 56°) plus 5 µl 1.0 M 2.(Morpholino)ethanesulfonic acid (MES) buffer, pH 5.0 or in 50 µl 100 mM Tris-base, pH 7.6 plus 2.5 µl 1.0 M MES buffer, pH 5.0. This resulted in solutions of pH 6.0. To 55 µl of the heat inactivated serum buffered at pH 6.0 were added 2 µl of pure liver alkaline phosphatase specific activity - 480 units/mg), 3 µl of neuraminidase and 3 µl of toluene as a

preservative. Several samples were incubated under these conditions for various lengths of time (24 hr, 48 hr, etc.). The maximum time the treatment was carried out was 96 hr. It was found that treatment was essentially complete after 72 hr as judged by the enzyme activity patterns on isoelectric focused gels (see section II.E above). Fresh aliquots of neuraminidase were added to the incubating mixture every twelve hr to insure continued activity of the neuraminidase throughout the incubation period.

CHAPTER III

PURIFICATION OF ALKALINE PHOSPHATASE

RESULTS AND DISCUSSION

A. Initial Extraction

Alkaline phosphatase is a glycoprotein located in the plasma membrane of mammalian tissues (See section I.B.2 above). Thus it is necessary to employ some type of extraction procedure to solubilize the enzyme so that it can be further purified. The method used here involves homogenization of the tissue in buffer and aqueous solubilization of the alkaline phosphatase by treatment with butanol. A summary of the specific activities and recoveries of the enzyme following each purification step is given in Tables 1 and 2.

After the alkaline phosphatase was extracted from the tissue with butanol it had a specific activity of approximately 0.2 - 0.3 U/mg (Tables 1 and 2). Fractionation steps with acetone and ammonium sulfate each produced approximately a 2 times purification of the enzyme with high yields to result in a crude extract with a specific activity of 1.0 U/mg. Both the fractionation

Table 1 Profile of human liver alkaline phosphatase purification by extensive purification techniques

Purification Fraction	Protein* (mg/ml)	Enzyme Activity (U/ml)	Specific Activity (U/mg)	Volume (ml)	Yield (%)	Purification (times)
Butanol Extract	8.8	2.28	.26	875	--	--
30-50% Acetone	9.4	5.49	.58	300	82	2.2
50-70% $(NH_4)_2SO_4$	16.4	19.3	1.18	84	81	4.5
DEAE-cellulose	2.3	8.68	3.77	100	43	14.5
ConA-Sepharose	.324	13.2	40.7	40	26	152
Sephadex G-200	.028	4.83	172	71	17	654
Pre-dialysate	.032	3.06	95.6			
50-65% $(NH_4)_2SO_4$	.052	4.68	90.0			
65-80% $(NH_4)_2SO_4$	.128	61.7	480		11	1850

The buffer for the first 4 steps was 10 mM Tris-base, pH 7.6 (HCl).
The buffer in the remaining steps was 100 mM Tris-base, pH 7.6 (HCl) plus 100 mM NaCl.
*Protein concentration was determined by the method of Lowry et al (1951).

Table 2 Profile of human liver alkaline phosphatase purification by original technique

Purification Fraction	Volume (ml)	Total Protein* (mg)	Total Activity (units)	Specific Activity (U/mg)	Yield (%)
Butanol Extract	1,600	71,600	10,289	.26	--
30-50% Acetone	--	--	8,578	--	80
50-70% $(NH_4)_2SO_4$	45	3,375	8,005	2.37	73
Sephadex G-200	25	226	5,325	23.56	52
DEAE-cellulose	3.75	22.5	2.270	100.8	22

The buffer used throughout the purification was 10 mM Tris-base, pH 7.6 (HCl)

*Protein concentration was determined from the OD_{280}, assuming 1 mg/ml has an OD_{280} of 1.00 (Warburg and Christian, 1941).

steps employing acetone or ammonium sulfate were easy, rapid steps in which the enzyme was immediately precipitated following addition of the acetone or ammonium sulfate.

Specific activities at this stage of purification are in agreement with other workers: Narayanan and Appleton (1972a) obtained a specific activity of 0.96 U/mg for human intestinal alkaline phosphatase after butanol extraction; 30-50% acetone fractionation and 50-70% ammonium sulfate fractionation steps; Smith et al (1968) obtained specific activities of 1.24 U/mg for human liver and 0.88 U/mg for human small intestine following the same procedures; Usategui-Gomez, Yeager and Tarbutton (1974) obtained a specific activity of 1.4 U/mg for an ammonium sulfate fraction of a butanol extract of human placental alkaline phosphatase.

B. Chromatography

1. General Considerations

Initially only two chromatographic steps were used: gel permeation on Sephadex G-200 and ion exchange on DEAE-cellulose according to Moss et al (1967). Chromatography of the ammonium sulfate fractions on the gel permeation column required the sample to be run in several aliquots because of the large volumes introduced when the extract was dialyzed at this stage in the purification.

The fractions from the gel permeation procedure were then applied to a column packed with DEAE-cellulose. This sequence of steps resulted in alkaline phosphatase extracts which consistently had specific activities of approximately 100 U/mg protein (See Table 2).

To help improve the purity of the enzyme affinity chromatography on concanavalin A-Sepharose (con A-Sepharose) was introduced into the purification procedure. This chromatographic step was employed initially after the ammonium sulfate fractionation step and before the gel permeation and ion exchange steps. However, this order was found to be disadvantageous because the con A-Sepharose column did not have enough glycoprotein binding capacity to handle the crude extract from the ammonium sulfate fractionation step. To alleviate this problem and also to enable the gel permeation step to be accomplished with only one sample application, the dialyzed ammonium sulfate fraction was first put on the ion exchange column, followed by con A-Sepharose affinity chromatography and finally gel permeation chromatography on Sephadex G-200. The ion exchange column as constructed could bind all the protein of the crude extract and the separation was not affected by the volume of sample applied. The con A-Sepharose column was now able to bind the alkaline phosphatase more efficiently. With the gel permeation step as the last

column procedure the sample could be run in one aliquot because the fraction from the affinity chromatography column was eluted in a single peak of low volume. The specific activities obtained by these modifications were 175 - 200 U/mg protein (Table 1), a considerable improvement over the original purification procedure.

2. DEAE-cellulose Ion Exchange

Ion exchange chromatography was performed using DEAE-cellulose equilibrated with the Tris-base buffer described in III.B.5 above. The size of the column was such that the protein applied: bed volume ratio was 1:10. Initially, the column was developed with a linear gradient of 0.0 - 0.3 M NaCl and the alkaline phosphatase activity was eluted at approximately 0.13 M NaCl. This is similar to results of Ohkubo, Langerman and Kaplan (1974) who found the rat liver alkaline phosphatase was eluted by approximately 0.14 M NaCl. In later purifications the gradient was made 0.0 - 0.2 M NaCl and better resolution of the protein peaks resulted. Figure 1 shows a typical elution profile of liver alkaline phosphatase from a DEAE-cellulose ion exchange column developed with 0.0 - 0.2 M NaCl gradient. The ion exchange chromatography step resulted in a 2 - 4 fold purification of the enzyme from all tissues (Tables 1 and 2). Smith et al (1968) obtained a 3-fold purification of liver and intestinal alkaline

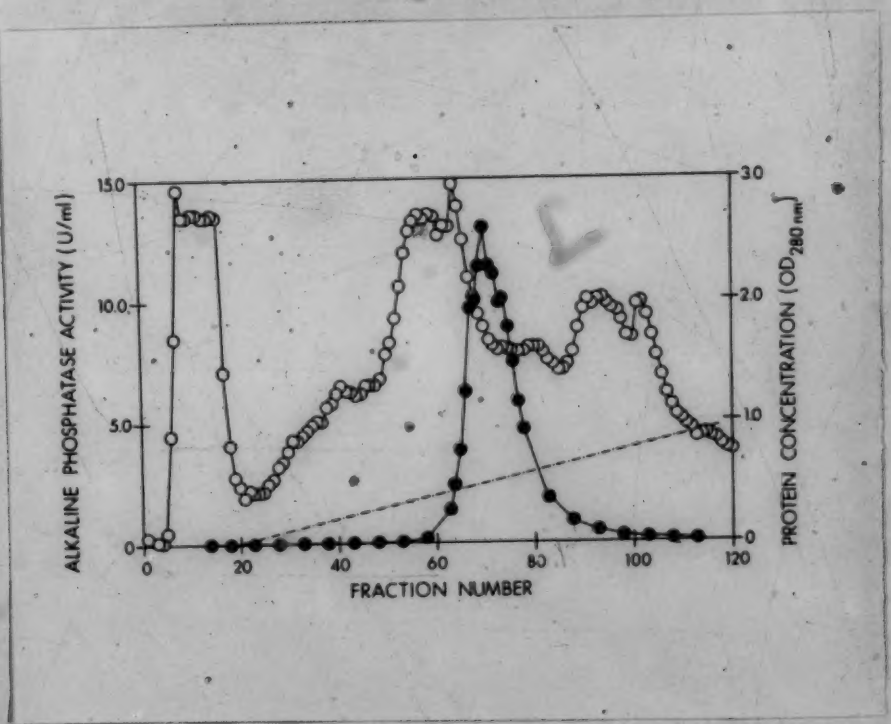

Figure 1 Profile on DEAE-cellulose ion exchange column chromatography of liver alkaline phosphatase. The column buffer was 10 mM Tris-base, pH 7.5 (HCl) containing 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. The gradient was from 0.0 - 0.2 M NaCl(Dashed line indicates the NaCl gradient). •, alkaline phosphatase activity (U/ml); o, protein concentration ($OD_{280\ nm}$).

phosphatase on a DEAE-cellulose column.

3. Concanavalin A-Sepharose

(a) Liver

Figure 2 shows a typical elution profile obtained when the DEAE-cellulose fractions were chromatographed by affinity chromatography on concanavalin A-Sepharose. No alkaline phosphatase could be eluted from the column with 2 column volumes of equilibrating buffer but the enzyme activity was recovered in good yield (Table 1) in a single peak with 100 mM mannose solution indicating that it is a loosely bound glycoprotein. Undoubtedly other loosely bound glycoproteins would be eluted as well. However, non-glycoproteins would be discarded in the buffer wash. The purification obtained with con A-Sepharose affinity chromatography was 10 - 30 times that of the applied fractions of liver alkaline phosphatase.

(b) Intestine

Over 65% of the intestinal alkaline phosphatase was eluted with the sample and wash buffer in a typical elution of intestinal alkaline phosphatase from the con A-Sepharose affinity chromatography column (Figure 3). The remaining 35% was eluted with 100 mM mannose solution. Lloyd (1970) suggested that a polysaccharide must have a minimum number of reactive end groups per mole in order to be bound by con A and Aspberg and Porath (1970) showed

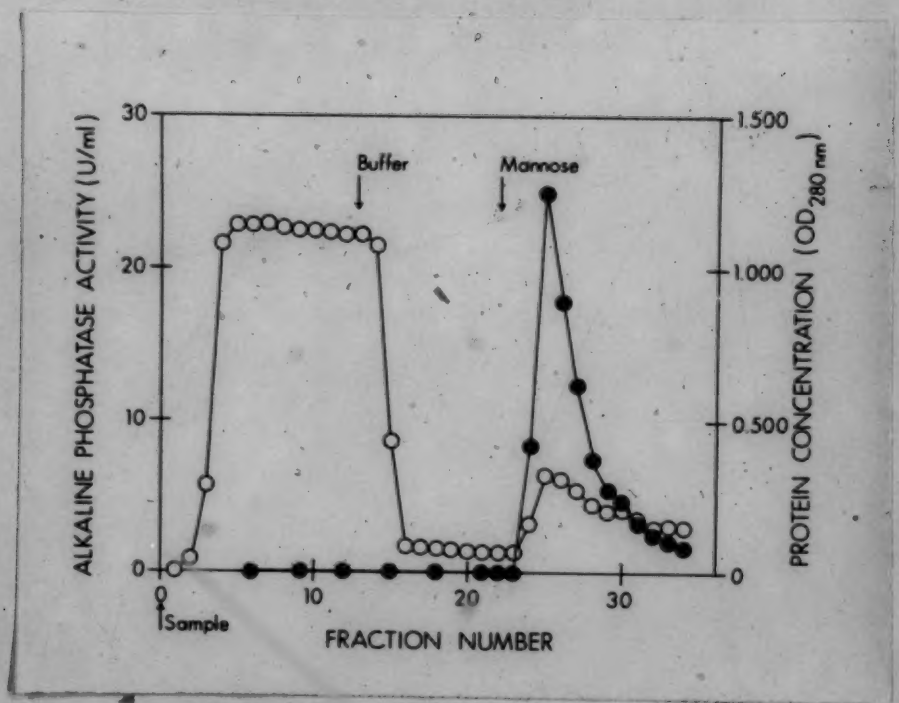

Figure 2 Elution profile of liver alkaline phosphatase from concanavalin A-Sepharose affinity chromatography. The column buffer was 100 mM Tris-base, pH 7.6 (HCl) containing 100 mM NaCl, 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. After the sample was applied, the column was washed with the column buffer and the alkaline phosphatase was eluted with 100 mM mannose in the column buffer solution. •, alkaline phosphatase activity (U/ml); o, protein concentration (OD_{280nm}).

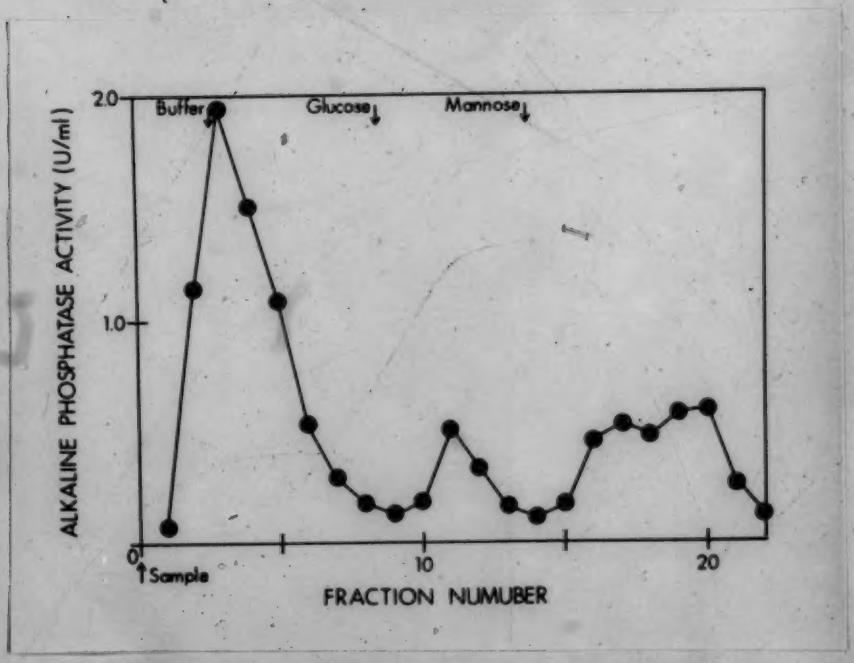

Figure 3 Elution profile of intestinal alkaline phosphatase from concanavalin A-Sepharose affinity chromatography. The column buffer was 100 mM Tris-base, pH 7.6 (HCl) containing 100 mM NaCl, 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. After the samples was applied the column was washed with the column buffer and the alkaline phosphatase was eluted with 100 mM mannose in the column buffer solution. •, alkaline phosphatase activity (U/ml).

that the carbohydrate to protein ratio was about eight times higher in the adsorbed materials on con A-Sepharose compared to the non-adsorbed material. Since intestinal alkaline phosphatase is not a sialoprotein as outlined in IV.D.4 below, it probably is a more weakly reacting species with respect to carbohydrate structure than liver alkaline phosphatase resulting in non-adsorption of the majority of the enzyme activity. The proportion of the intestinal alkaline phosphatase that was adsorbed must have contained enough carbohydrate residues of a reactive nature or configuration to be adsorbed to the con A-Sepharose column. Keeping in mind that the con A-Sepharose column was not overloaded with glycoprotein this may suggest that the intestinal alkaline phosphatase may not be homogenous with respect to carbohydrate moieties or their configuration.

(c) Bone

A typical elution profile of bone alkaline phosphatase (Figure 4) from the con A-Sepharose affinity chromatography column shows that 25% of the activity was not adsorbed to the column. The rest was eluted with 100 mM mannose solution. The column was not overloaded with respect to glycoprotein. This suggests that a minor portion of bone alkaline phosphatase may contain a more weakly reacting species with respect to carbohydrate

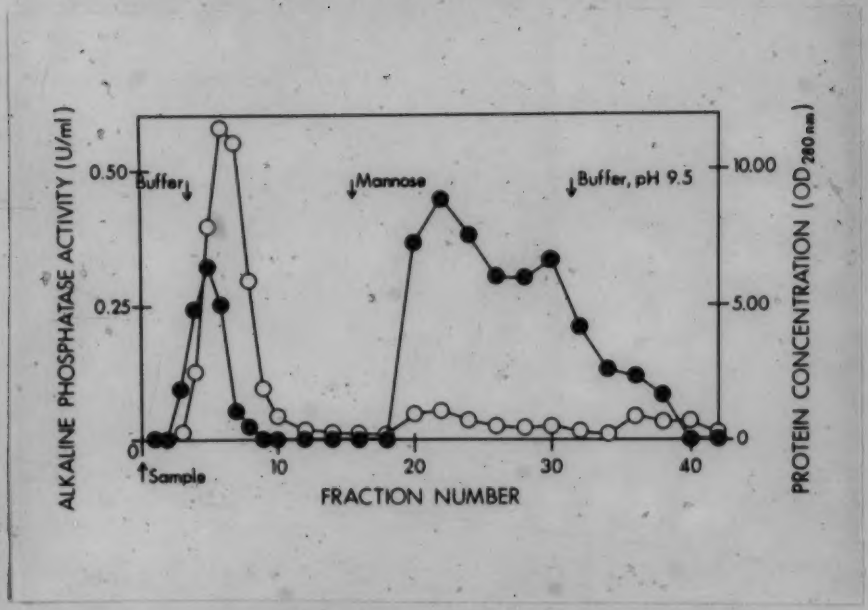

Figure 4 Elution profile of bone alkaline phosphatase from concanavalin A-Sepharose affinity chromatography. The column buffer was 100 mM Tris-base, pH 7.6 (HCl) containing 100 mM NaCl, 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. After the sample was applied, the column was washed with the column buffer and the alkaline phosphatase was eluted with 100 mM mannose in the column buffer solution. •, alkaline phosphatase activity, (U/ml); o, protein concentration ($OD_{280\ nm}$).

structure as discussed under intestine (See section III.B. 3(b) above). This is indicative that bone alkaline phosphatase may not be homogenous with respect to its carbohydrate moieties, similar to the pattern shown for intestinal alkaline phosphatase, but that the major component, that which was adsorbed, behaves in a manner more similar to that of liver alkaline phosphatase.

4. Sephadex G-200 Gel Permeation

The purification obtained from the Sephadex G-200 column was from 3 - 11 times depending on whether it was the initial chromatographic step or if it followed another chromatographic step (Compare Tables 1 and 2). Different preparations of liver alkaline phosphatase chromatographed on the Sephadex G-200 gel permeation column consistently were eluted with the same elution volumes, indicating they were homogeneous with respect to molecular size (Figure 5). More than half of the bone and intestinal extract activities, when put through the Sephadex G-200 column were not included to any degree and were eluted in the void volume. This is indicative of a molecular size greater than 800,000 daltons which may be an aggregate of the molecular species that eluted within the fractionation range of the gel permeation column (See section IV. B.1 below).

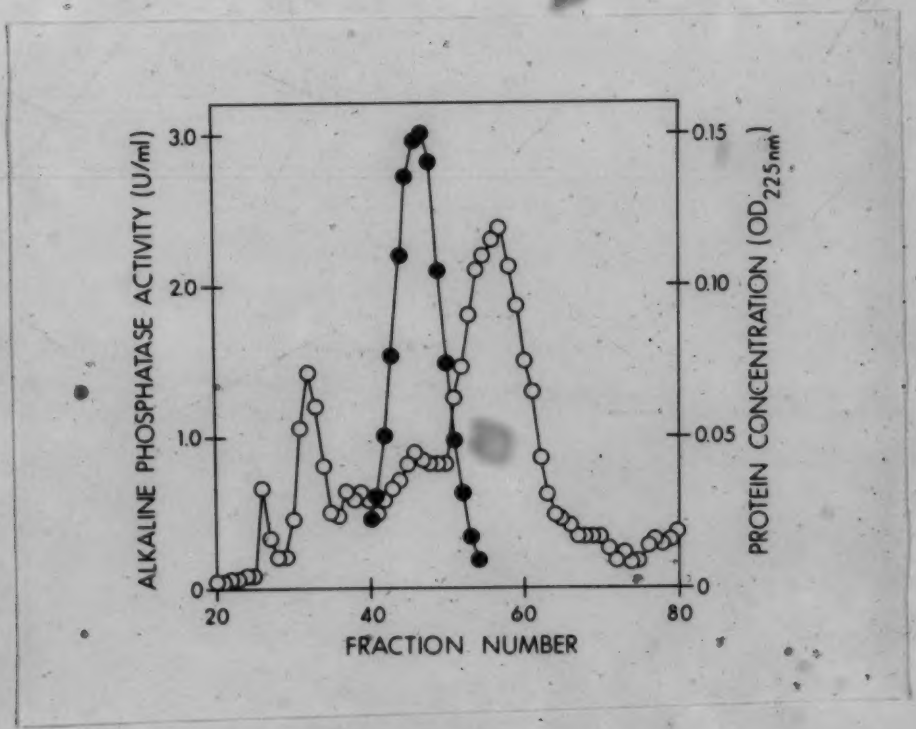

Figure 5 Elution profile of liver alkaline phosphatase from Sephadex G-200 gel permeation column chromatography. The column buffer was 100 mM Tris-base, pH 7.6 (HCl) containing 100 mM NaCl, 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. •, alkaline phosphatase activity; o, protein concentration ($OD_{225\ nm}$).

The yields obtained after the gel permeation step for the liver isoenzyme were approximately 15% of the enzyme present in the original butanol extracts and the specific activities were 175 - 200 U/mg protein. Other workers using the same techniques except the con A-Sepharose column have obtained similar yields: Smith et al (1968) obtained a 10.4% yield for the human intestinal enzyme; Usategui-Gomez, Yeager and Tarbutton (1974) obtained an 11% yield for human placental alkaline phosphatase; Narayanan and Appleton (1972a) obtained a 5% yield for human intestinal alkaline phosphatase.

C. Ammonium Sulfate Fractionation of Liver Alkaline Phosphatase

The liver isoenzymes of alkaline phosphatase were fractionated further with ammonium sulfate to remove additional impurities and any protein which may have denatured during the preparation. One third of the enzyme activity came down in the 65% (w/v) saturated solution of ammonium sulfate (4.1 M is saturation at 25°) and the rest was precipitated by an 80% saturated solution of ammonium sulfate. This fractionation resulted in a protein in the 65 - 80% saturated ammonium sulfate pellet which had a specific activity for alkaline phosphatase of 480 U/mg protein; This was 3 times purer than the enzyme obtained off the gel permeation column and resulted in a total purification of 1850 times that of the original butanol extract.

D. Purity and Stability of Liver Alkaline Phosphatase

The purity of the liver alkaline phosphatase preparation was demonstrated by examination of the protein stained and enzyme stained bands obtained on polyacrylamide disc gel electrophoresis (See section IV.A.1 below). What appeared to be a single protein staining band had the same mobility as the alkaline phosphatase activity band (Figure 6). The major band on SDS polyacrylamide disc gels (See section IV.B.2 below) was determined to be alkaline phosphatase protein on the basis of the correlation obtained between the intensity of the major protein band and the specific activities obtained from various preparations.

The most purified alkaline phosphatase was run on an SDS polyacrylamide gel and it was stained for protein using Coomassie Blue R-250 (Figures 14 and 15) as outlined in II.H.3 above. This gel was then scanned at 620 nm using a Beckman DU spectrophotometer equipped with a Gilford Model 2410-5 linear transport for scanning gels. From the scan of this gel as well as from visual examination of the gel itself, it was estimated that the major protein band attributed to alkaline phosphatase was approximately 75% pure. This estimation of purity is subject to error with respect to differential staining of the proteins by Coomassie Blue as well as the inherent error in the estimation itself. However, this is the first report that

demonstrates the purity of human liver alkaline phosphatase preparation. The specific activity of this sample was 480 U/mg protein. By these criteria specific activities of 175 - 200 U/mg protein obtained for samples carried through the gel permeation step but not through the final ammonium sulfate fractionation indicate that they are approximately 30% alkaline phosphatase protein and that 100% pure alkaline phosphatase protein from human liver would have a specific activity of 650 U/mg protein. From the activity obtained in the original butanol extract, it was found that the pathologically normal human liver contained an average of 5.6 U/g wet weight of tissue. For a 1.5 kg liver this results in a total of 8400 U of alkaline phosphatase activity or 12 mg of alkaline phosphatase protein. Alkaline phosphatase represents 0.0009% of the protein present in the human liver compared to 6% for E. coli alkaline phosphatase protein. (See section I.G. above). However, a 15% yield of alkaline phosphatase after the purification procedure results in obtaining just under 2 mg alkaline phosphatase protein from 1.5 kg liver tissue.

Table 3 shows the data obtained from the long term stability trial done on samples of the purified liver alkaline phosphatase. It was found that either 10% glycerol added to the sample which was in 10 mM Tris-base buffer, pH 7.6 (HCl) and maintained at 4° or freezing the

Table 3 Stability trial of liver alkaline phosphatase under various storage conditions.

Storage Conditions	Initial Activity	3 hr	9 days	19 days	29 days	50 days	78 days	92 days	113 days
Condition 1	.94	1.03	1.14	1.02	1.29	1.34	1.25	1.29	1.19
Condition 2	1.00	1.09	1.10	.95	1.07	1.04	.80	.74	.60
Condition 3	1.06	.99	.98	.92	1.29	1.71	1.06	1.22	1.06
Condition 4	1.00	1.15	1.14	1.19	1.23	1.27	1.17	1.20	1.31
Condition 5	.62	.29	.21	.21	.19	.26	.17	.10	--

The dilutions in the various buffers were such that the initial enzyme activity was set at 1.00 U/ml. The buffer conditions were: 1. 10 mM Tris-base, pH 7.6 plus 10% glycerol at 4°; 2. 100 mM Tris-base, pH 7.6 plus 10% glycerol at 4°; 3. 10 mM Tris-base, pH 7.6 plus 50% glycerol at 4°; 4. 10 mM Tris-base, pH 7.6 at -20°; 5. 10 mM Tris-base, pH 7.6 plus 80% $(NH_4)_2SO_4$ at 4°.

sample in 10 mM Tris-base buffer, pH 7.6 (HCl) provided the best conditions for the storage of the enzyme. Precipitation of the enzyme in an 80% saturated solution of ammonium sulfate resulted in a rapid loss of alkaline phosphatase activity and is not recommended for storage of the enzyme.

E. Concentration of Purified Alkaline Phosphatase

1. Polyethylene Glycol

Samples of liver tissue and bone tissue alkaline phosphatase were dialyzed against at 10% solution of polyethylene glycol, 20,000 (Carbowax) in 10 mM Tris-base buffer, pH 7.6 (HCl) that contained 0.1 mM $MgCl_2$ and 0.02 mM $ZnCl_2$. This resulted in concentration of the enzyme solution to one tenth of its original volume in 7 - 9 hr with 90% of the enzyme activity recovered. This technique has been used to concentrate samples from the Sephadex G-200 column which were typically as much as 50 ml. It was of little use for trying to concentrate samples with volumes less than 1 ml.

2. Membrane Concentrators

The Minicon-B ultrafilter concentrator (Amicon Corp.) is a membrane concentrator that was used to concentrate purified liver alkaline phosphatase. The sample, 13.3 ml containing 22.5 U of enzyme activity, was concentrated to 1.3 ml containing 10.6 U of enzyme activity.

This was only a 47% recovery of the enzyme after concentration, although the activity was concentrated 5 times. The reason for this loss of activity was probably due to non-specific binding of enzyme molecules to the membrane. As a result these membrane concentrators cause a considerable loss of enzyme activity and are unsuitable for quantitative recoveries.

CHAPTER IV

STUDIES OF ALKALINE PHOSPHATASE

RESULTS AND DISCUSSION

A. Electrophoretic Properties of Alkaline Phosphatase

1. Polyacrylamide Disc Gel Electrophoresis

Disc gel electrophoresis was carried out mainly for the purpose of demonstrating the purity of the human liver alkaline phosphatase (Discussed under section III.D above) and not for distinguishing between the isoenzymes of various tissue origins. Figure 6 shows the electrophoretic patterns of human liver alkaline phosphatase stained for protein and alkaline phosphatase activity. All the fractions from the various stages in the purification procedure showed only one activity band with the same mobility as the activity and protein bands obtained with the most pure enzyme. Several attempts were made to stain a polyacrylamide gel with periodic acid Schiff reagent for glycoprotein. A few of these gels showed a faint pink band with the same mobility as the protein and alkaline phosphatase activity bands. Unfortunately, this band was too light to be successfully photographed.

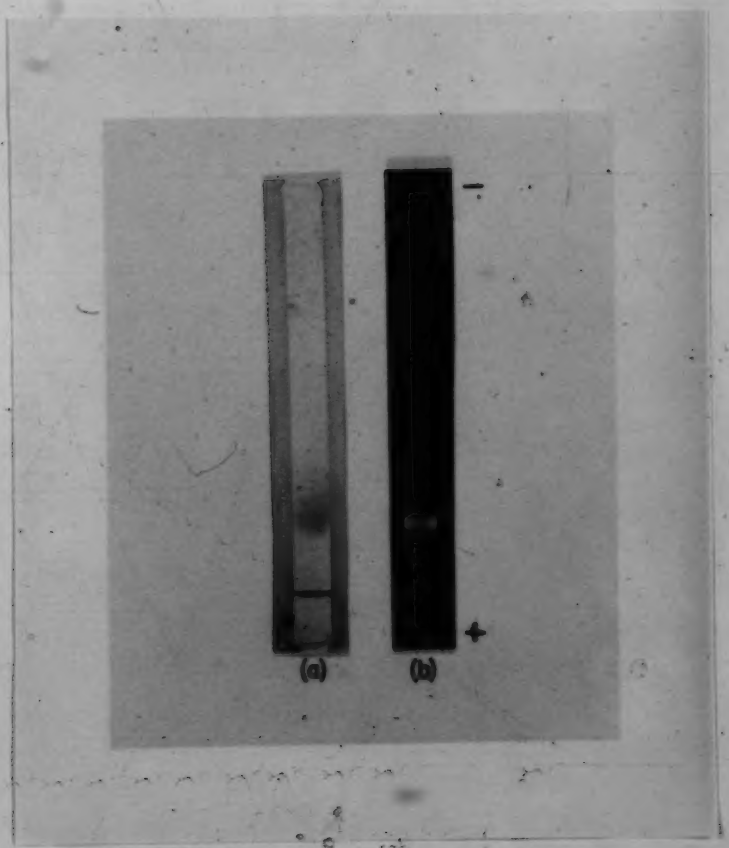

Figure 6 Polyacrylamide disc gel electrophoresis of liver alkaline phosphatase stained for protein and enzyme activity. The gels were 5.5% polyacrylamide. The samples were liver alkaline phosphatase stained (a) with Coomassie Blue R-250 for protein, (b) with naphthol AS-MX phosphoric acid for enzyme activity.

The liver extract was electrophoresed with a serum[10] reported to have increased liver alkaline phosphatase. Figure 7 shows that liver alkaline phosphatase from either serum or tissue origin had the same electrophoretic mobility on polyacrylamide disc gels. The tissue enzyme, however, seemed to migrate in a sharper band than did the enzyme from a serum, but perhaps there were more components to the serum enzyme than could be separated by electrophoresis (See section IV.A.2.(a)below). Often with serum samples there was activity at the top of the gel that did not enter the gel. This activity could be the result of an aggregate of alkaline phosphatase which was unable to penetrate the polyacrylamide gel matrix (See section IV.A.2. (c) below).

Electrophoresis was performed to compare the migration of alkaline phosphatase from bone tissue with that from liver (Figure 8). The liver tissue extract formed a single activity band, whereas, the bone extract showed a broad smear of enzyme activity with a slower mobility than that of the activity from the liver extract,

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10 Serum samples denoted liver serum and bone serum refer to the results obtained by agarose electrophoresis and heat denaturation as performed and interpreted by the Department of Laboratory Medicine, University of Alberta Hospital.

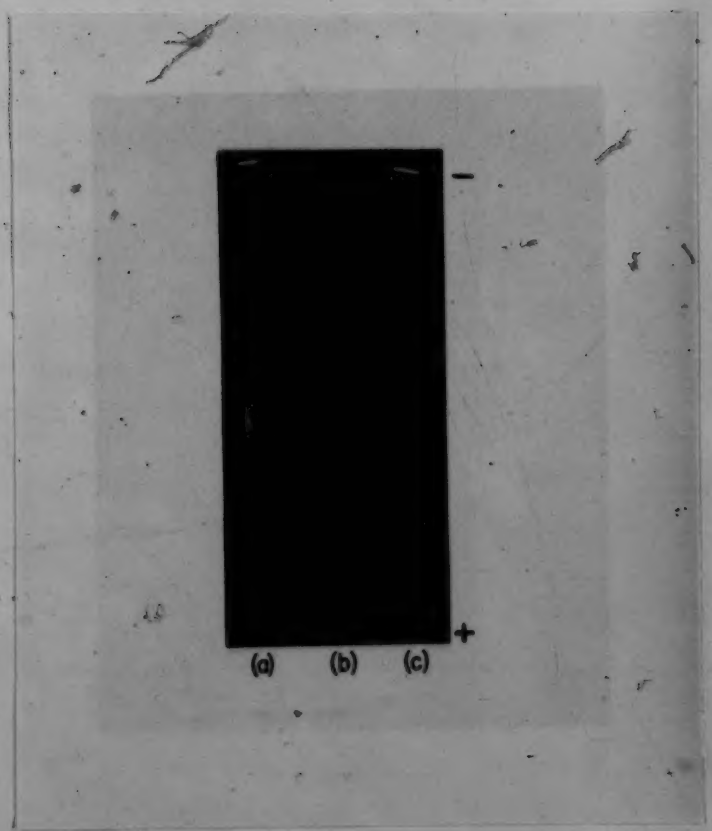

Figure 7 Polyacrylamide disc gel electrophoresis of liver tissue and liver serum alkaline phosphatase. The gels were 5.5% polyacrylamide. The samples stained with naphthol AS-MX phosphoric acid were: (a) liver serum alkaline phosphatase, (b) liver tissue alkaline phosphatase, (c) mixture of liver serum and liver tissue alkaline phosphatase.

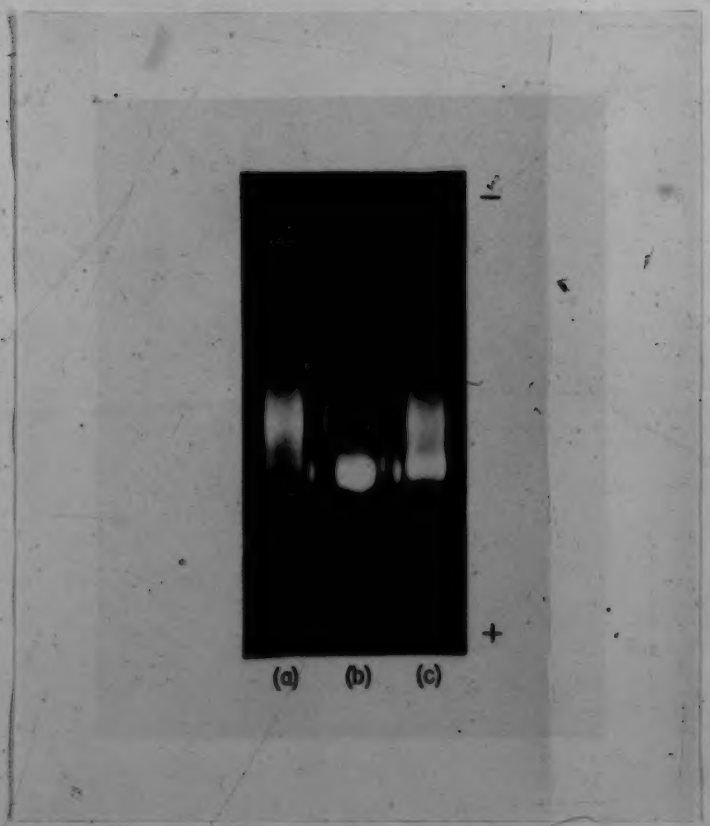

Figure 8 Polyacrylamide disc gel electrophoresis of liver tissue and bone tissue alkaline phosphatase. The gels were 5.5% polyacrylamide. The samples stained with naphthol AS-MX phosphoric acid were: (a) bone alkaline phosphatase, (b) liver alkaline phosphatase, (c) mixture of bone and liver alkaline phosphatase.

although there was a partial overlap of the activity zones. A mixture of both the liver and the bone extracts (Figure 8(c)) showed two bands of activity although they were not distinctly separated. The bone isoenzyme band seems to be a broad trailing band electrophoresing adjacent to the liver isoenzyme making polyacrylamide disc gel electrophoresis a less than perfect technique for the separation of these isoenzymes of alkaline phosphatase. Studies by other workers on cellulose acetate (Rhone, White and Gidaspow, 1973; Fritsche and Adams-Park, 1972) and polyacrylamide disc gels (Dingjan, Postma and Stroes, 1973; Walker and Pollard, 1971) showed the same patterns of separation of the alkaline phosphatase isoenzymes by electrophoresis. The bone and liver isoenzymes were not completely distinguishable and interpretation relied on isoenzyme specific tests, such as heat denaturation, to establish the relative amounts of the isoenzymes present (See section IV.D.2 below for results of heat stability studies).

2. Isoelectric Focusing on Polyacrylamide Gels

(a) Properties of the Isoenzymes

In order to obtain clearer separations of the isoenzymes of alkaline phosphatase than was possible on electrophoresis the use of isoelectric focusing on polyacrylamide gels was investigated. It was found that 4%

polyacrylamide gels that contained 1% pH 4-6 ampholine plus 1% pH 3-10 ampholine gave the best separation and distribution of the alkaline phosphatase isoenzymes throughout the gel (Figure 9). As well, the gradient obtained with this ampholine mixture was linear throughout the length of the gel (Figure 10).

(i) Liver

Liver alkaline phosphatases from tissue and sera were found to focus in two bands at the acidic end of the gel. Isoelectric points were determined as outlined in section III.I above in which the pH of the isoelectric focused band was obtained by relating the R_f of that band to graph of pH plotted against relative mobility (Figure 10). The lower band, pH_I 4.0, was associated with liver tissue extracts as well as pathological sera with increased alkaline phosphatase due to liver disease[10]. The upper liver band, pH_I 4.2, was the predominant band seen in normal sera and would appear to be the same as the 'slow' liver band migrating in the α_2 region on cellulose acetate electrophoresis as reported by Rhone and Mizuno (1972) and Rhone et al (1973). These authors further demonstrate the presence of a 'fast' liver band migrating in the α_1 region on cellulose acetate electrophoresis in some serum specimens. The lower band seen on isoelectric focusing which was determined to have an isoelectric point of 4.0 and

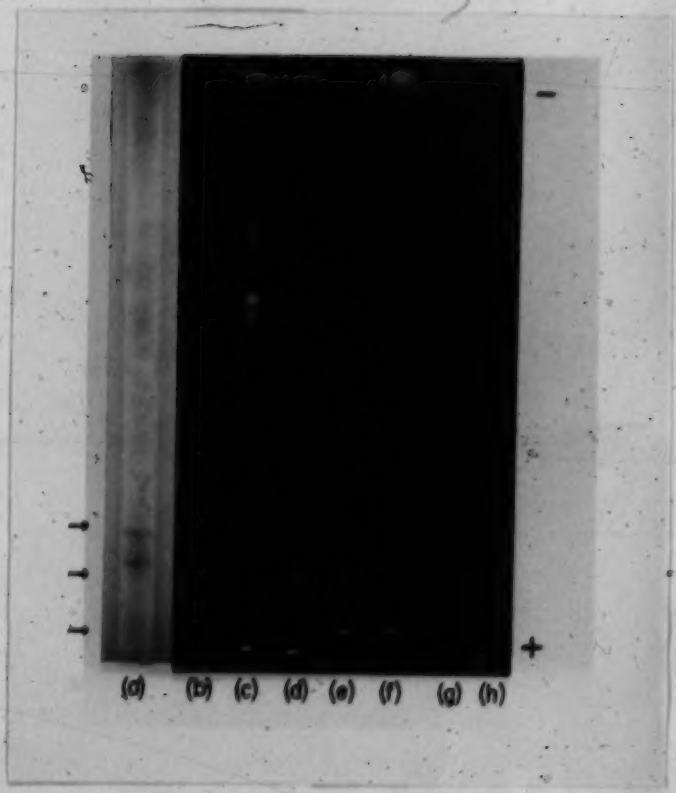

Figure 9 Isoelectric focusing on polyacrylamide disc gels of tissue and serum samples of alkaline phosphatase. The gels were 4% polyacrylamide containing 1% pH 4-6 ampholine and 1% pH 3-10 ampholine. The alkaline phosphatase samples were: (a) 75% pure liver enzyme, (b) 75% pure liver enzyme, (c) bone tissue extract in a liver serum, (d) liver tissue in a liver serum, (e) bone serum, (f) liver serum, (g) bone tissue in 1% Triton X-100, (h) bone tissue. Gel (a) was stained for protein with Coomassie Blue R-250 and gels (b) -(h) were stained for activity with naphthol AS-MX phosphoric acid.

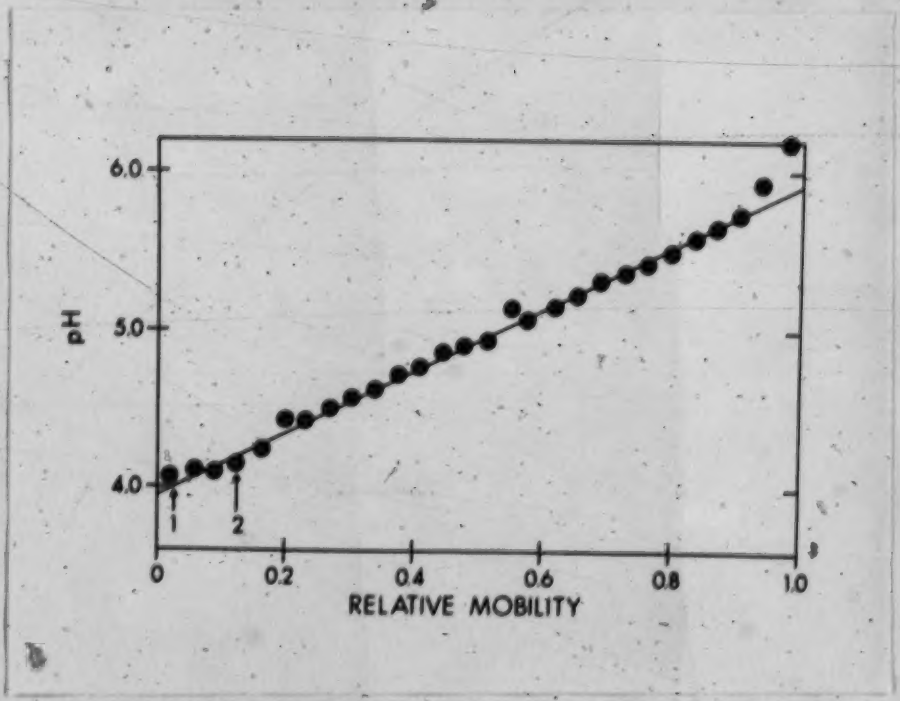

Figure 10. A pH gradient obtained from a polyacrylamide disc gel subjected to isoelectric focusing. The gel was 4% polyacrylamide containing 1% pH 4-6 ampholine and 1% pH 3-10 ampholine. Liver tissue alkaline phosphatase focused at position 1; liver serum alkaline phosphatase focused at position 2.

which could be found in liver tissue extracts and in pathological sera associated with liver disease, would, therefore, appear to be the same as this 'fast' liver band seen on electrophoresis. Isoelectric focusing separates these two liver bands very distinctly. These isoelectric points are in agreement with those obtained by other workers: pH_I 4.3 - 4.6 for baboon liver (Hammond et al, 1973), pH_I 3.9 for human liver (Usategui-Gomez et al, 1974), pH_I 3.8 for human liver (Greene and Sussman, 1973).

Attempts to obtain an isolectric focused gel of the pure liver isoenzyme stained for protein, in order to show a band coincident with the activity band as was done for electrophoresis (See section IV.A.1 above) were unsuccessful. There were no Coomassie Blue stainable bands evident in the region where the alkaline phosphatase focused on the gel, although one band was sometimes seen approximately 40% down the gel and always a smear was evident at the top of the gel. With the highly purified liver extract (specific activity 480 U/mg protein), two faint protein bands could be seen on the gel immediately above the region of liver alkaline phosphatase activity (Figure 9). These protein bands were felt to be due to alkaline phosphatase protein that had been altered in some way. It may be due to aggregation of the enzyme as discussed in IV.A.2 (c) below or perhaps the ampholine

had a deleterious effect on the alkaline phosphatase molecule so that its isoelectric point was altered and the protein inactivated. It was found that if a given amount of specimen was applied to an ordinary standard polyacrylamide gel and electrophoresed, that a faster and more intense development of product fluorescence occurred than when that same amount of specimen was applied to a polyacrylamide gel containing ampholine and isoelectric focusing carried out. Ampholine itself or some artefact of isoelectric focusing definitely has some retarding effect on the activity of alkaline phosphatase. Smith, Lightstone and Perry (1971) suggest that during isoelectric focusing the isoenzymes are in an acid medium for an appreciable time and this may explain why the enzyme is less active than on standard polyacrylamide gels.

(ii) Bone

The bone tissue extracts did not always focus in clear distinct bands on isoelectric focused gels. Often enzyme activity was seen in a smear near the top of the gel. Again this may be due to aggregation of the alkaline phosphatase molecules as discussed in IV.A.2. (c) below. Bone tissue extracts that were freshly prepared and electrofocused resulted in clear activity patterns on the gel and a series of five bands was obtained - a band in the same place as the normal serum band (i.e. liver isoenzyme)

pH_I 4.2, three bands immediately above this, pH_I 4.3, 4.4 and 4.5 and the smeared band near the top of the gel, pH_I 5.5. The isoelectric focusing technique results in the appearance of several bands of activity associated with the bone isoenzyme compared to only one diffuse band on electrophoresis. One possible explanation for these multiple bands could be that they are due to variations in the sialic acid content (See section IV.A.2 (b) below). When the bone extract was aged at 4° for several days and then focused, the pH_I 5.5 activity smear increased in intensity, whereas, the other bands decreased in intensity. Storing the bone extracts with 1.0% (v/v) Triton X-100 would retard the process of aggregation for at least 72 hr.

A bone tissue extract was focused with a liver serum sample[10] (Figure 9(c)) in order to determine where the bone alkaline phosphatase focused with respect to the liver. Little success was obtained with this approach, as the bone bands focused in the top half of the polyacrylamide gel. However, when a bone serum specimen was run alongside a bone extract the two samples showed the same bands of activity.

(iii) Intestine

Fewer studies were done on the intestinal and placental isoenzymes because there is more clinical interest in distinguishing between the liver and bone isoenzymes.

Intestinal alkaline phosphatase focused in two close bands of activity, pH_I 4.7 and 4.8, immediately above the group of three bands associated with the bone isoenzyme activity. The placental isoenzyme focused in two bands, pH_I, 4.5 and 4.6 which were nearly coincident with the upper band of bone activity. However, placental alkaline phosphatase is not of diagnostic interest since it becomes elevated only after the third trimester of pregnancy and can be distinguished from the bone isoenzyme by means of the heat stability test (See section IV.D.2 below).

(b) The Effect of Neuraminidase on Isoelectric Focusing

Neuraminidase (from Vibrio cholerae) cleaves terminal sialic acid residues from protein molecules and thereby increases their isoelectric points. The isoelectric points of the isoenzymes of alkaline phosphatase from liver and bone were increased in a series of discrete steps until an equilibrium activity pattern was established (Figure 11). This seems to suggest that each alkaline phosphatase band seen on electrofocused gels represents an enzyme of different sialic acid composition. The final isoelectric points of the two zones of activity obtained from desialated human liver alkaline phosphatase were pH_I's 6.6 and 6.8 and were the same, within experimental error, as those for the neuraminidase treated bone alkaline phosphatase. This suggests a basic similarity in the

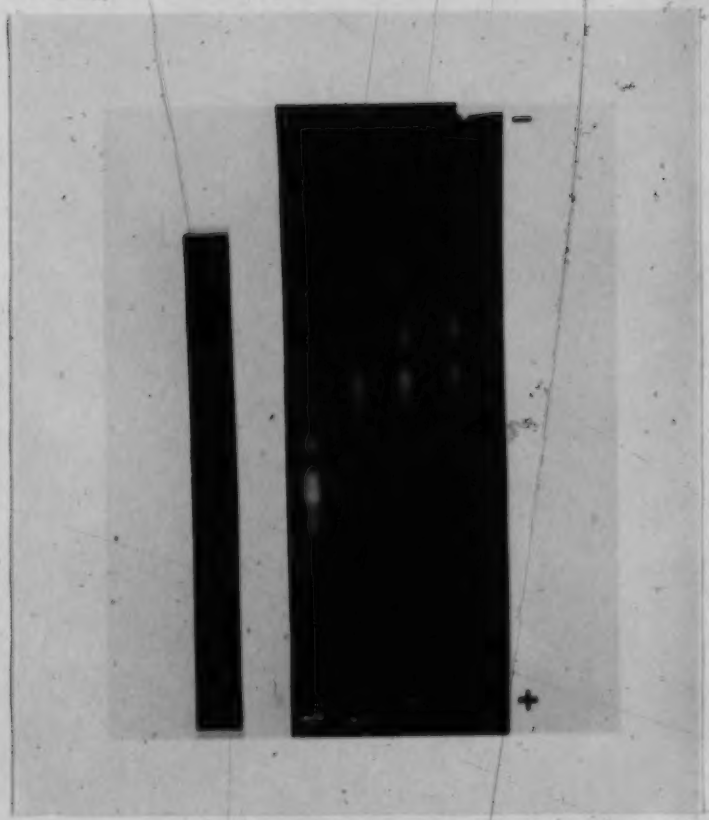

Figure 11 Isoelectric focusing on polyacrylamide disc gels of liver alkaline phosphatase which was treated with neuraminidase. The gel on the left was 4% polyacrylamide containing 1% pH 4-6 ampholine and 1% pH 3-10 ampholine; the gels on the right were 4% polyacrylamide containing 1% pH 5-8 ampholine and 1% pH 7-9 ampholine. From left to right the samples were liver tissue alkaline phosphatase treated with neuraminidase for 0 hr, 24 hr, 48 hr, 72 hr, 96 hr.

structure of alkaline phosphatase from these two tissue sources. Neuraminidase treated alkaline phosphatase from both liver and bone retained full enzymatic activity. It was not fully understood why neuraminidase treatment resulted in two bands of equal activity after equilibrium had been established. This same behavior was seen with α_1-acid glycoprotein, a single polypeptide chain, which could be resolved into seven variant protein preparations after desialation by mild acid hydrolysis (Schmid et al, 1973). The authors suggested this variance was due to different numbers of amino acid replacements on the protein molecule.

(c) Aggregation of Alkaline Phosphatase

(i) General Considerations

Serum samples that were stained for enzyme activity and purified liver alkaline phosphatase that was stained for protein (Figure 9) on electrofocused gels showed bands or smears at the top of the gel which did not penetrate the polyacrylamide matrix. These bands may be due to aggregation of the alkaline phosphatase molecules.

The aggregate seen in serum samples was active with respect to alkaline phosphatase activity and was likely a result of bile alkaline phosphatase which was formed in the liver. When a specimen of bile was electrofocused a very intense band was seen at the top

of the gel as well as a fainter band in the region of the intestinal alkaline phosphatase. This would support the concept (Price and Sammons, 1974) that the bile alkaline phosphatase is an aggregated molecule associated with lipoprotein. Recently, Price, Hill and Sammons (1972) have described a high molecular weight isoenzyme of alkaline phosphatase from bile which was bound to a phosphatidylcholine complex and was retained at the origin on starch gel electrophoresis.

In contrast to the serum 'aggregate' the 'aggregate' seen with the purified liver enzyme did not possess alkaline phosphatase activity. Such aggregation may lead to increased molecular stability because of decreased surface area per unit weight of the 'aggregate' resulting in a more favorable charged environment within the 'aggregate'. It is not unlikely that a membrane enzyme, when extracted, may seek this type of stability. Treatment of purified enzyme with 1 mM β-mercaptoethanol seemed to disrupt the 'aggregate' and caused some of the protein of an early sample to enter the gel and focus in two bands near the bottom of the gel associated with the enzyme activity. β-Mercaptoethanol prevents disulfide bond formation and thereby may discourage aggregation. Treatment of the enzyme with pure human serum albumin also resulted in the appearance of a faint band that focused

coincident with the single enzyme activity band obtained with a liver extract. Perhaps albumin acted to stabilize the alkaline phosphatase protein by contributing ionic stability to the molecule so that it did not seek aggregation for 'protection'.

Studies with Triton X-100 treated bone alkaline phosphatase support these same conclusions. The non ionic detergent did not affect the activity of bone alkaline phosphatase but caused the activity smear near the top of the gel, thought to be due to an 'aggregate', to be diminished in intensity and the activity in the region of the lower bands as were described in IV.A.2 (a).(ii) above to be enhanced. Fritsche and Adams-Park (1974) found that all isoenzymes of alkaline phosphatase present in sera could not be resolved on polyacrylamide (6.5%) gel electrophoresis, but that a fraction was always retained at the origin following electrophoresis. Also, Jennings et al (1970) describe a high molecular weight enzyme in sera that is retained at the origin of starch gel support media and that this origin band disappears after treatment with butanol. This behavior towards butanol and Triton X-100 supports the postulate that the serum 'aggregate' may be a lipoprotein complex as suggested by Dunne, Fennelly and McGeeney (1967). This is in agreement with a postulate of Fishman (1974) which states that con-

sidering the detergent action of Triton X-100, it may be expected that alkaline phosphatase in sera may be associated with hydrophobic regions of other proteins and with alkyl chains of lipids in the membranes from which it was derived.

(ii) Sample Application Technique on Electrofocused Gels and Aggregation Phenomenon

A concentrated extract of purified liver or bone alkaline phosphatase underwent aggregation at a slower rate than a diluted form of the enzyme. It seems that these isoenzymes tend to aggregate in dilute solution in order to achieve molecular stability. The aspects of sample preparation and application for electrofocusing of the tissue extracts were investigated. Because of the highly concentrated activity involved in the extracts, dilutions had to be made to apply to the gels an amount of activity that was in line with that found in a serum sample. The same samples diluted in different buffers or for different lengths of time prior to sample application or in different volumes of buffer showed different activities towards the activity stain after the electrofocusing procedure. For example, dilution in AMP buffer enhanced the alkaline phosphatase staining activity compared to dilution in Tris buffer; older dilutions developed fluorescence faster than fresh dilutions; samples applied in 10 µl showed more staining activity,

perhaps due to less tendency to aggregate, than samples applied in 150 µl. It is interesting to note that all these samples had the same p-nitrophenyl phosphatase activity. This would suggest that the aggregated form of the enzyme seen at the top of the gel was present in the sera in the amount that existed when it was drawn and was not formed after it left the body. The reasons for these effects are not completely understood. However, from these observations the following precautions were noted: all specimens should be treated in the same manner for comparison of activity, all dilutions should be as fresh as possible and the smallest volume possible should be applied to the gels and this should be consistent for all specimens.

B. Molecular Weight Determination of Human Liver Alkaline Phosphatase

1. Gel Filtration Chromatography

(a) Molecular Weight of Native Alkaline Phosphatase

Sephadex G-200 is a dextran polymer formed from the fermentation of sucrose resulting in a polymer of glucose residues linked by α-1,6-glucosidic bonds with α-1,3-glucosidic bonds as branch points.

Each of the molecular weight markers and the purified liver alkaline phosphatase were chromatographed a series of six times on the Sephadex G-200 column employing 100 mM Tris-base, as the column buffer and the molecular

weight of the enzyme was determined from the plot shown in Figure 12. The apparent molecular weight of 220,000 (range: 199,000 - 226,000) was obtained for human liver alkaline phosphatase. Similar results have been obtained by other workers using gel filtration chromatography: Smith et al (1968) using 10 mM Tris-base, 100 mM NaCl buffer obtained a molecular weight of 220,000 and Moss et al obtained a molecular weight of 225,000.

A second molecular weight determination was done on the Sephadex G-200 column using 50 mM triethanolamine (TEA) buffer, plus 100 mM NaCl. The standards and the alkaline phosphatase were chromatographed and the molecular weight obtained was 213,000 (range: 204,000 - 220,000). This value supports the determination done on the 100 mM Tris, 100 mM NaCl buffered column.

Initially the molecular weight determinations were carried out in a low ionic strength buffer of 10 mM Tris-base. On this column the elution patterns of all the standards and the alkaline phosphatase, each of which was chromatographed a series of eight times, were considerably altered and a molecular weight of 146,000 (range: 139,000 - 151,000) was obtained. When this result was compared to those of Smith et al (1968) who obtained a molecular weight of 220,000 and Moss (1970) who obtained a molceular weight of 225,000, it was felt that the low ionic strength buffer resulted in non-specific adsorption of the protein to the

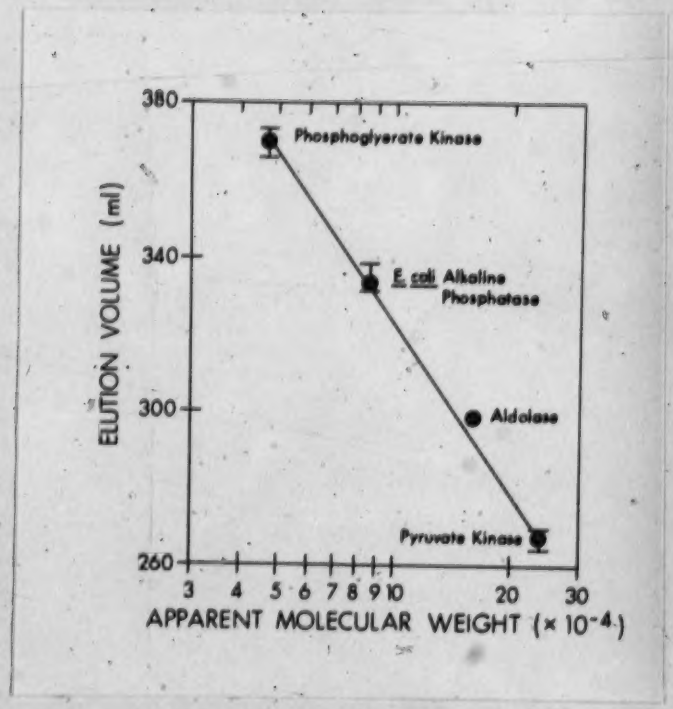

Figure 12 Standard graph of the molecular weight markers used for Sephadex G-200 molecular weight determination. The column buffer was 100 mM Tris-base, pH 7.6 (HCl) containing 100 mM NaCl and 0.1 mM $MgCl_2$.

gel matrix causing it to be retarded and resulting in a low apparent molecular weight. Thus, the column buffer was changed to the high ionic strength 100 mM Tris, 100 mM NaCl buffer and molecular weight determinations were then performed in that system.

Subsequent to these molecular weight determinations for alkaline phosphatase using Sephadex G-200 gel filtration, the subunit molecular weight was determined to be 76,700 using sodium dodecyl sulfate polyacrylamide gels (See section IV.B.2 below). This result is in agreement with the subunit molecular weight for rat liver alkaline phosphatase of 75,000 obtained by Ohkubo et al (1974). These authors further showed rat liver alkaline phosphatase to have a molecular weight of 154,000 by sedimentation equilibrium analysis. In considering the subunit molecular weight obtained for liver alkaline phosphatase it would appear that perhaps the apparent molecular weight of 220,000 obtained on the 100 mM Tris, 100 mM NaCl buffered Sephadex G-200 column may be higher than the real molecular weight. The high ionic strength buffer used to determine this molecular weight could perhaps lead alkaline phosphatase, a hydrophobic membrane enzyme, to aggregate in order to protect itself against this ionic environment. This would accelerate its movement through the gel permeation column and tend to increase the molecular weight.

(b) Effect of Neuraminidase Treatment on the Molecular Weight of Alkaline Phosphatase

Treatment of alkaline phosphatase with neuraminidase removes the sialic acid groups from the enzyme molecule and a subsequent molecular weight determination on this desialated enzyme resulted in a molecular weight of 188,000 (range: 175,000 - 193,000). This change in molecular weight from 220,000 before neuraminidase treatment to 188,000 after treatment was probably due to more than the weight of the removed sialic acid residues alone. Perhaps cleavage of the negatively charged sialic acid residues allows an increase in non-specific adsorption of the enzyme to the Sephadex G-200 matrix. This would retard the elution of alkaline phosphatase and decrease its apparent molecular weight. Alternately, the larger molecular weight of 220,000 could be in error as a result of slight aggregation occurring during chromatography. Removal of the terminal sialic acid residues may alleviate this tendency to aggregate and hence the desialated protein has an apparent lower molecular weight. The subunit molecular weight of 76,700 determined by SDS gel electrophoresis (See section IV.B.2 below) is in better agreement with the latter explanation in that a dimer molecular weight of 153,400 is closer to the value 188,000 determined by gel filtration.

2. Subunit Molecular Weight Determination by Sodium Dodecyl Sulfate

The apparent subunit molecular weight of human liver alkaline phosphatase was found to be 76,700 (S.D. 2,800) by SDS polyacrylamide gel electrophoresis against standard proteins (Table 4). Glycoproteins containing more than 10% carbohydrate behave anomalously during SDS polyacrylamide gel electrophoresis when compared to standard proteins due to decreased SDS binding per gram of glycoprotein compared to the standard proteins (Segrest and Jackson, 1972). By increasing the polyacrylamide concentration in the gels, molecular sieving becomes the predominant electrophoretic force over charge and this results in the higher molecular weight values asymptotically approaching values close to the real molecular weight. Seven determinations of the subunit molecular weight of alkaline phosphatase were performed in polyacrylamide disc gels using concentrations ranging from 6.5% to 10.5% polyacrylamide (Figures 13 - 15). The mobility of one of the standard proteins, phosphorylase a, was thought to behave anomalously at higher polyacrylamide concentrations (Figure 13) and so it was not weighed in determining the standard graph. The anomalous behavior found with glycoproteins by Segrest and Jackson (1972) was not seen with the glycoprotein alkaline phosphatase. Instead, the molecular weight results varied randomly over the polyacryl-

Table 4 Results of subunit molecular weight determination on sodium dodecyl sulfate polyacrylamide gels

Polyacrylamide Concentration % (w/v)	Initial Determination	Second Determination
6.5	77,500	74,000
8.0	80.500	72,000
9.5	78,000	--
10.0	78,000	--
10.5	--	77,000

The average molecule obtained from these seven determinations was 76,700. The standard deviation was 2,800.

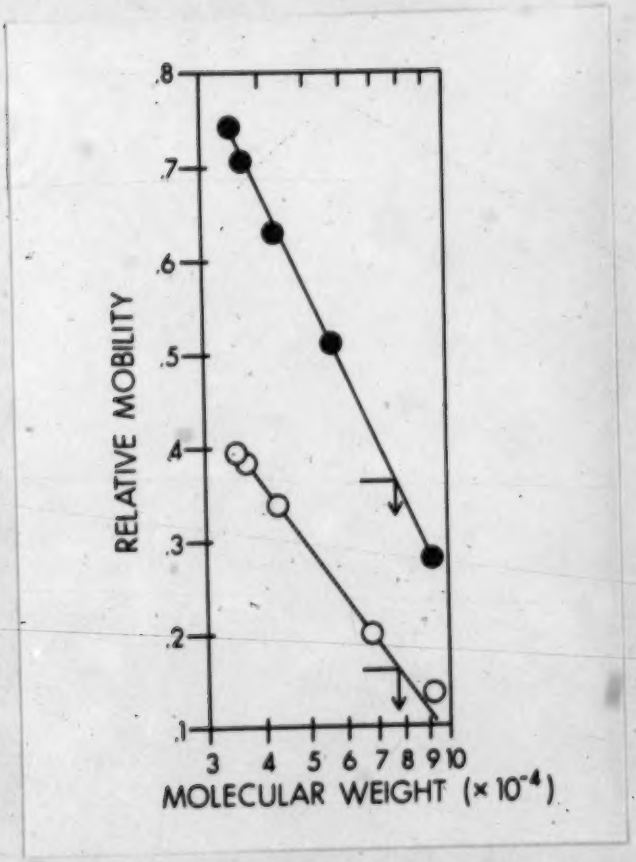

Figure 13 Standard graph of the molecular weight markers used for the SDS polyacrylamide disc gel subunit molecular weight determination. The molecular weight markers were phosphorylase a, human serum albumin, glutamate dehydrogenase, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase (See text for molecular weight values). Arrow indicates mobility and molecular weight obtained for liver alkaline phosphatase. ●, 6.5% polyacrylamide gel; ○, 10.5% polyacrylamide gel.

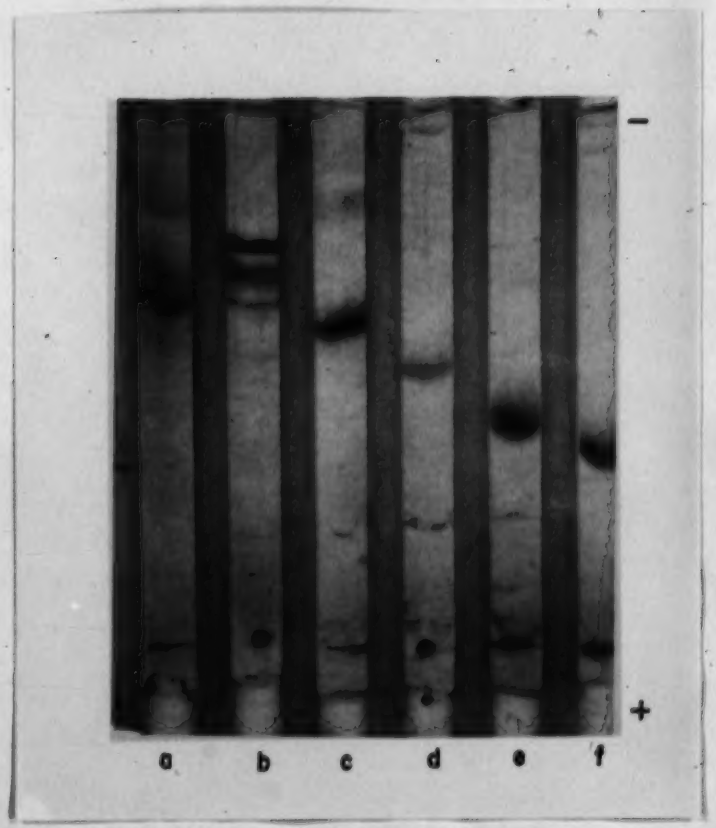

Figure 14 6.5% polyacrylamide disc gel electrophoresis of the standards used for sodium dodecyl sulfate molecular weight determination. The samples from left to right were: (a) liver alkaline phosphatase, (b) phosphorylase a, (c) human serum albumin, (d) glutamate dehydrogenase, (e) ovalbumin, (f) glyceraldehyde-3-phosphate dehydrogenase.

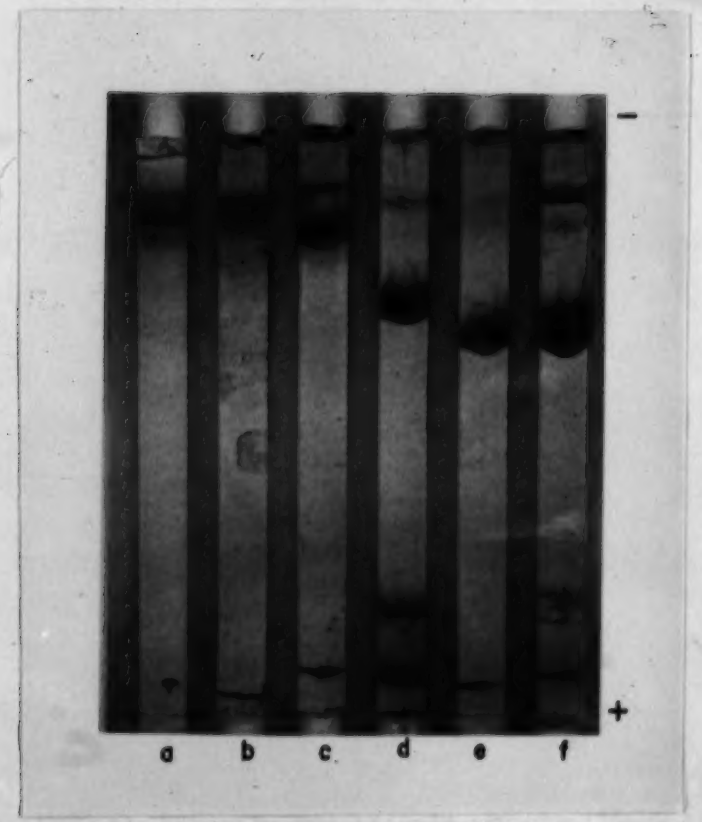

Figure 15 10.5% polyacrylamide disc gel electrophoresis of the standards used for sodium dodecyl sulfate molecular weight determination. The samples from left to right were: (a) liver alkaline phosphatase, (b) phosphorylase a, (c) albumin, (d) ovalbumin, (e) glyceraldehyde-3-phosphate dehydrogenase, (f) lactate dehydrogenase.

amide range tested (Table 4). Perhaps the reason for this digression of alkaline phosphatase from the behavior exhibited by other glycoproteins was that it has a higher molecular weight than the glycoproteins tested by Segrest and Jackson (1972) and its mobility is limited by molecular sieving even in 6.5% polyacrylamide gels.

C. Substrate Specificity Studies on the Isoenzymes of Alkaline Phosphatase

The substrate specificities were determined for the isoenzymes of alkaline phosphatase. The absolute activity in international units obtained with each enzyme source with p-nitrophenyl phosphate as substrate was then set at 100 and the absolute activities of that enzyme with each of the other substrates were expressed relative to this value with p-nitrophenyl phosphate (Table 5). Thus, the data show, for example, that for purified liver alkaline phosphatase p-nitrophenyl phosphate is the most rapidly hydrolyzed substrate followed by β-naphthol phosphate, β-glycerophosphate and phenolphthalein monophosphate. The specific activity of the enzyme with p-nitrophenyl phosphate as substrate would be 14 times that with phenolphthalein monophosphate as substrate. The bone and liver tissue enzymes react with similar activities with each of the four substrates. Intestinal and placental tissue extracts on the other hand show twice the activity with

Table 5 Relative activities of the isoenzymes of alkaline phosphatase with four different substrates.

Isoenzyme	p-nitrophenyl phosphate	phenolphthalein monophosphate	β-glycero-phosphate	β-naphthol phosphate
Liver	100	7.1	10.7	26.6
Bone	100	7.3	9.9	23.2
Intestine	100	14	9.4	28
Placenta	100	14	36.7	33
Liver Serum	100	7.9	24.4	31.9
Bone Serum	100	8.3	40.5	55.1

The value obtained for p-nitrophenyl phosphate activity was set at 100 for each isoenzyme and the activities with the other substrates were expressed relative to this value.

phenolphthalein monophosphate than do liver and bone isoenzymes and the placental tissue extract shows 4 times the activity with β-glycerophosphate than do the other 3 tissue extracts. Bone and liver serum samples show the same specificities with p-nitrophenyl-phosphate and phenolphthalein monophosphate as the liver and bone tissue extracts do, but increased activity towards β-glycerophosphate and β-naphthol phosphate. This discrepancy between the reactivities of the serum samples and those of the tissue extracts is not fully understood. The increased activities of the liver and bone serum samples towards these two substrates could be a result of the components of serum. Alternately, it could be a result of other phosphatase activities. These results illustrate the need to use a substrate which will not prejudice the value of the total enzyme activity because of an increased level of any one of the isoenzymes. However, if one of the isoenzymes of alkaline phosphatase was significantly more reactive towards a particular substrate, then perhaps that substrate could be utilized to aid in the determination of the proportion of that particular isoenzyme in a mixture of the isoenzymes. From the results shown in Table 5, β-glycerophosphate may be useful in this sense to selectively differentiate the placental isoenzyme which is 4 times as reactive towards this substrate as are the other 3 isoenzymes.

The isoenzymes associated with liver and bone have the most importance clinically in the determination of the alkaline phosphatase isoenzymes for diagnosis. It would therefore be desirable that these two isoenzymes have the same affinity for a particular substrate. From these studies it was concluded that β-glycerophosphate and β-naphthol phosphate would not meet these criteria because of the greater increase of the placental isoenzyme with these substrates and the disimilar reactivities of the liver serum and bone serum specimens shown towards these substrates. Also, these two substrates involve phosphate determinations after incubation with the enzyme which is a lengthly and insensitive procedure in the range of phosphate concentrations released by normal serum alkaline phosphatase activities. Thus methods that utilize either p-nitrophenyl phosphate or phenolphthalein monophosphate as a substrate are preferred. The liver and bone isoenzymes have the same reactivities towards either substrate whether as tissue extracts or serum samples and the intestinal and placental isoenzymes show twice the activity of the liver and bone speciments towards phenolphthalein monophosphate. Both of these substrates involve simple analysis procedures, either direct spectrophotometric measurement after the reaction has been stopped or the reaction can be measured kinetically with a recording spectrophotometer. The greater specific

activity of the isoenzymes towards p-nitrophenyl phosphate, however, make it the preferred method for assaying alkaline phosphatase.

D. Miscellaneous Studies on Alkaline Phosphatase

1. K_m determination of p-Nitrophenyl Phosphate for Alkaline Phosphatase

The K_m of p-nitrophenyl phosphate for human liver alkaline phosphatase was found to be 5.0×10^{-4} M. The Lineweaver-Burk plot (Figure 16) drawn from the data in Table 6 gave a value of $-1/K_m$ of -2.0 mM^{-1} which resulted in the K_m of 5.0×10^{-4} M. In order to compare the activities of the various samples of alkaline phosphatase using p-nitrophenyl phosphate the substrate concentration should be at least $20 \times K_m$. To ensure zero order reaction kinetics and complete saturation of the enzyme over a wide range of enzyme concentrations, the assay medium used throughout the present work contained 1×10^{-2} M (10 mM) p-nitrophenyl phosphate, or 20 times the K_m. Eaton and Moss (1968) obtained a Michaelis constant of 8×10^{-4} M at pH 10.5 for p-nitrophenyl phosphate using human liver alkaline phosphatase and Harkness (1968) obtained a Michaelis constant of 8×10^{-4} M for p-nitrophenyl phosphate at pH 10.5 using human placental alkaline phosphatase.

2. Heat Stability of the Isoenzymes of Alkaline Phosphatase

The differences in heat stability of the various isoenzymes of alkaline phosphatase have been described by

Table 6 Data obtained for the K_m determination of p-nitrophenyl phosphate for human liver alkaline phosphatase

p-Nitrophenyl Phosphate Concentration (mM)	Velocity (U/ml)	1/[S] (mM^{-1})	1/v ($units^{-1}$)
10.0	161	0.1	.0062
1.0	105	1.0	.0095
.33	59.7	3.0	.0168
.20	46.5	5.0	.0215
.14	35.8	7.1	.0279
.11	30.0	9.1	.0333
.095	27.7	10.5	.0361

The buffer used was 0.782 M AMP, pH 10.3 containing 1.5 mM $MgCl_2$ and the enzyme assays were performed on a recording spectrophotometer at 30°.

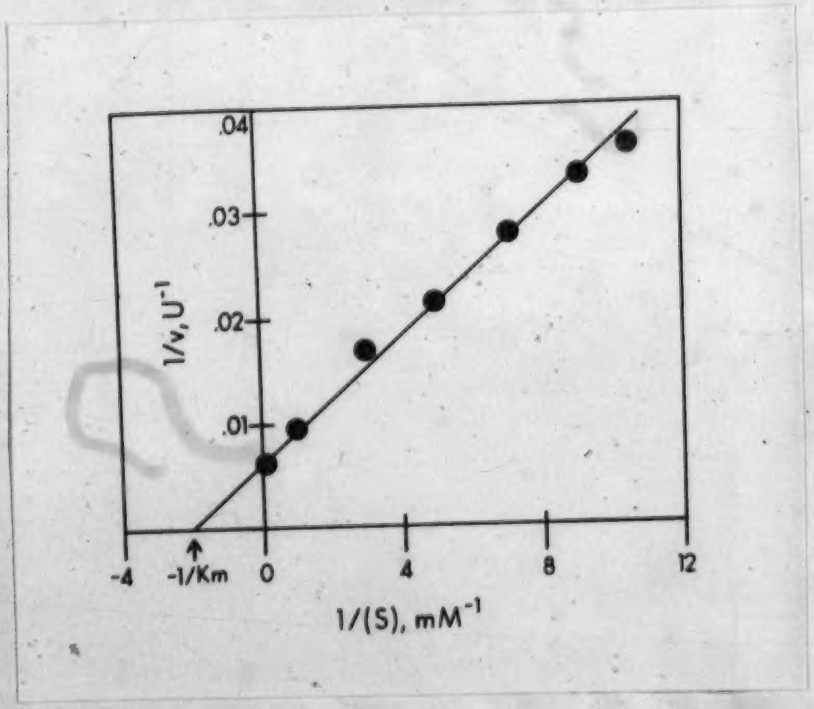

Figure 16 Lineweaver-Burk plot used for K_m determination of p-nitrophenyl phosphate for liver alkaline phosphatase. The assay buffer was 0.782 M AMP, pH 10.3 (HCl) containing 1.5 mM $MgCl_2$. The value of $-1/K_m$ was -2.0 mM^{-1} measured at 30°.

Posen et al (1965) who incubated the enzyme samples at 56° for 10 min. and have been used in differentiating the isoenzymes present in sera. Heat stability trials (Table 7) done on the four tissue isoenzymes of alkaline phosphatase: bone, intestine, liver and placenta support the results obtained by Posen et al (1965). The bone isoenzyme was the least resistant to heat; as only 26% of its activity remained after incubation for 10 min. at 56°. The placental enzyme was the most heat stable with 92% of its activity remaining after the heat treatment. The heat stability for the liver and intestine isoenzymes were 74% and 85% respectively. Using the criterion of heat stability a serum sample which is largely inactivated by heat treatment, is said to be predominantly bone alkaline phosphatase and a serum sample which is not denatured severely by heat treatment may contain predominantly any of the other isoenzymes. These isoenzymes can partially be distinguished by electrophoresis (See section IV.A.1 above).

Table 7 Heat stability determination on the isoenzymes of alkaline phosphatase.

Isoenzyme		Activity Remaining after Treatment (%)	Average of Trials for each Isoenzyme (%)
Bone	Trial 1	29	
	Trial 2	32	26
	Trial 3	19	
Intestine	Trial 1	88	
	Trial 2	94	85
	Trial 3	72	
Liver	Trial 1	76	
	Trial 2	78	74
	Trial 3	69	
Placenta	Trial 1	87	
	Trial 2	92	92
	Trial 3	98	

Each trial consisted of incubating the isoenzyme at 56.0° for 10 min. and assaying for remaining alkaline phosphatase activity. The activity is expressed as percent of the activity before treatment.

GENERAL DISCUSSION AND CONCLUSIONS

The availability of pure alkaline phosphatase protein will allow the study of specific phosphatase activities reported to be associated with the enzyme as well as characterization of the protein itself. In this study human liver alkaline phosphatase was purified for the purpose of studying the latter. The purity of the enzyme was estimated at 75% as judged by polyacrylamide gel electrophoresis. This is an 1850 times purification over that of the butanol extract and it resulted in a protein with a specific activity of 480 U/mg protein. This is the first report that demonstrates the purity of a human liver alkaline phosphatase preparation.

Isoelectric focusing was used to determine the isoelectric points of liver, bone, intestinal and placental alkaline phosphatase in relation to the isoelectric points of the components found in normal and pathological sera. This is the first report of the isoelectric points of human bone and intestinal alkaline phosphatase. The focusing technique was found to be superior to electrophoresis for the separation of the four isoenzymes of alkaline phosphatase. With this technique a normal serum containing predominantly "slow" liver alkaline phosphatase was distinguishable from a serum with a large amount of "fast" liver alkaline phosphatase; also, liver serum alkaline phos-

phatase was distinguishable from bone serum alkaline phosphatase.

The molecular weight of human liver alkaline phosphatase was determined using gel filtration chromatography to be 220,000 daltons. This value is consistent with those of other workers, however, it may be erroneously high. With this consideration in mind it appears reasonable that the subunit molecular determined on SDS-polyacrylamide gels to be 76,700 is one half of the entire enzyme molecule. It is suggested that human liver alkaline phosphatase is a dimer molecule of two identical molecular weight subunits. This has not been previously demonstrated for human liver alkaline phosphatase.

Substrate specificity studies were performed to determine a substrate which was not greatly influenced by the presence of any particular isoenzyme of alkaline phosphatase in a serum sample. It is known that certain of the isoenzymes of alkaline phosphatase are more reactive with particular substrates. For comparison of the substrates the same buffer, pH and temperature conditions were used with each substrate, a point which had not always been adhered to in previously performed comparisons. It was shown that liver and bone tissue alkaline phosphatase showed parallel reactivities with the four substrates examined but that all tissues on the whole showed a higher

specific activity with p-nitrophenyl phosphate than with phenolphthalein monophosphate. The use of p-nitrophenyl phosphate was therefore concluded as the preferred substrate for assaying alkaline phosphatase from various tissue sources.

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